Remarks

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendments, claims 14-36 are pending in the application, with claims 14 and 32 being the independent claim. These changes are believed to introduce no new matter, are well supported throughout the specification, and their entry is respectfully requested. Support for new claims 33-36 can be found in the specification, at, *e.g.*, page 16, lines 26-29. Based on the above amendments and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Objection

At page 3, first paragraph, of Paper No. 13, the Examiner objects to claim 11 as containing non-elected embodiments (*e.g.*, an endothelial cell and a macrophage).

As claim 11 was canceled in the Amendment filed September 20, 2002, Applicants presume this objection was intended for claim 28. Claim 28 has been amended to delete the non-elected embodiments without prejudice or disclaimer of the subject matter therein. Withdrawal of this objection is respectfully requested.

Rejections Under 35 U.S.C. § 112, First Paragraph

The Examiner rejects claims 14-32 under 35 U.S.C. § 112, first paragraph, because, according to the Examiner, the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims. *See,* Paper No. 13, page 3.

The Examiner cites several grounds for rejecting the claims for non-enablement. Applicants respectfully traverse each of these grounds below.

The Legal Standard for Enablement

The initial burden of proving that a specification is non-enabling is on the Examiner. It is axiomatic that a specification is presumed to be enabling unless the Examiner provides acceptable objective evidence or sound scientific reasoning showing that it would require *undue* experimentation for one of ordinary skill in the art to make and use the claimed invention.

Specifically, the court in *In re Marzocchi*, 169 U.S.P.Q. 367, 369 (CCPA 1971) stated:

> [A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of § 112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

It is well-established that some experimentation is permitted, so long as it is not "undue." See, *In re Angstadt*, 190 USPQ 214, 219 (CCPA 1976); *United States v.*

Telectronics, Inc., 8 USPQ2d 1217, 1222 (Fed. Cir. 1988). In determining whether undue experimentation would be required to practice a claimed invention, several relevant factors have been identified. These factors include: (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3), the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims. *In re Wands*, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). Thus, whether undue experimentation is needed is not a single, simple factual determination, but rather is a conclusion reached by weighing many factual considerations.

Further, "as concerns the breadth of a claim relevant to enablement, the only concern should be whether the scope of enablement provided to one skilled in the art by the disclosure is commensurate with the scope of protection sought by the claims." M.P.E.P. § 2164.08.

In Vivo Delivery of a Genetic Vector

The Examiner asserts that the full scope of the claims is not enabled because "the broad claims encompass any route of delivering a vector comprising a nucleotide molecule encoding FPGS into neoplastic cells *in vivo* (including both systemic and local delivery)," and that "vector targeting *in vivo* to desired cells or tissues, for this instance neoplastic cells, continues to be unpredictable and inefficient." *See,* Paper No. 13, pages 5-6. In support of this position, the Examiner summarizes passages from four references to selectively emphasize the technical difficulties associated with delivering vectors to target cells *in vivo* (Dang, C.V., *et al.*, *Clin. Cancer Res. 5*:471-474 (1999); Miller, N. and Vile, R., *FASEB J.*

9:190-199 (1995); Deonarian, M.P., *Exp. Opin. Ther. Patents 8*:53-69 (1998); and Verma, I.M. and Somia, N, *Nature 389*:239-242 (1997)).

Applicants assert that these references merely set forth technical hurdles that need to be overcome in order to *increase the efficiency* with which genetic vectors are targeted to cells *in vivo*. They do not, however, indicate that vector delivery to target cells is impossible or infeasible, but rather, that it is not ideal and that there is room for improvement. As of 2001, there were 400 approved clinical gene therapy trials, with over half relating to cancer. Greco and Dachs, *J. Cell. Physiol. 187*:22-35 (2001) (of record). Satisfying the enablement requirement does not require the claimed invention or aspects of the claimed invention to be in optimum or perfect form, so long as the claimed invention can be made and used without undue experimentation. Clearly, the existence of approximately 400 clinical gene therapy trials indicates that numerous gene delivery techniques are being used successfully, despite the admitted need for improvements in vector design.

Deonarian, in fact, describes experimental results in which genetic vectors were successfully delivered to liver cells *in vivo* using ligand-targeted receptor-mediated endocytosis of polyplexes:

> Reporter gene delivery experiments *in vivo* showed 85% of the injected DNA was taken up by the liver by 10 min. A great deal of research has followed, including *in vivo* gene delivery of albumin to rats with LDL receptor deficiency. An average of 1000 copies of the plasmid were found per hepatocyte resulting in a level of 34 μg/ml human albumin in the serum of animals 2 - 4 weeks after injection and partial hepatectomy.

Deonarian at 59, left column. At page 13 of Paper No. 13, the Examiner contends that Deonarain does not teach that the injected DNA molecule could be delivered efficiently to

neoplastic cells other than the liver, and that the claims are not drawn to killing neoplastic cells in a liver.

First, Applicants' claims do not exclude the sensitization or killing of liver cancer cells. Second, Applicants are merely citing Deonarain as an exemplary publication where gene delivery was successful without requiring direct inoculation.

The Dang reference presents the report of a meeting on gene therapy and translational cancer research. Several gene therapy approaches were discussed, including, immunotherapy, expression of tumor suppression genes, and oncogene inactivation. Cancer gene therapy in the context of suicide genes was not even discussed in Dang. Similarly, the article by Verma and Somia discusses the challenges in gene therapy in a very broad sense, without regard for the type of gene therapy (*e.g.*, suicide gene therapy; replacement of a mutated or defective gene), or the type of disease (*e.g.*, genetic, acquired) being treated. Thus, the references cited by the Examiner do not substantiate a *prima case* of non-enablement of the claimed invention, which relates to a particular approach to gene therapy (*i.e.*, suicide gene therapy, involving the expression of the FPGS gene in combination with anti-folate drugs--a well-known class of chemotherapeutic drugs).

In Paper No. 13, the Examiner cites two additional papers relating to gene directed enzyme/prodrug cancer therapy, Xu *et al.*, *Clin. Cancer Res. 7*:3314-3324 (2001) and Greco and Dachs, *J. Cell. Physiol. 187*:22-35 (2001). The Examiner states that these papers "also note that the lack of an efficient gene delivery to target cells still remains a major problem to attain therapeutic efficacy as for all other areas of gene therapy." Paper No. 13, page 7.

Regarding the Xu paper, Applicants submit that the sections marked "GDEPT" and "VDEPT" are both relevant to the claimed invention and include numerous citations of

positive reports, as well as ways to improve current delivery systems. Regarding the Greco and Dachs paper, although the authors conclude that some problems need to be solved before these strategies become "routinely adopted in the clinic," they go on to state: "nevertheless, the results described in this review and their clinical application are encouraging, and illustrate both feasibility and future promise for GDEPT as a cancer treatment." *See*, Greco and Dachs (2001), conclusion.

On pages 7, last three lines, to page 9, first two lines, of Paper No. 13, the Examiner discusses the embodiment where the non-viral vector is in the form of a cationic liposome. The Examiner asserts that the use of any cationic liposome for systemic or intravenous delivery of a transgene for *in vivo* applications is limited and unpredictable with regard to obtaining therapeutic effects and cites select passages from three references for alleged support (Scherman *et al., Current Opinion in Biotechnology 9*:480-485 (1998), Romano *et al., Stem Cells 18*:19-39 (2000), and Filion *et al., Int. J. Pharm. 162*:159-179 (1998)).

Applicants respectfully submit that the reported disadvantages or possibility of adverse effects with any particular gene delivery system (such as, *e.g.*, cationic liposomes or DNA-protein complexes) certainly does not rise to a level of inoperativeness. As stated above, the available gene delivery systems are not perfect and can benefit from improvements as research ensues. That is not to say, however, that these systems cannot be made and used by those skilled in the art using routine experimentation. For instance, Romano states:

> Recent studies have reported significant success in improving the *in vivo* performance of nonviral gene delivery systems [citations omitted]. These improvements have been achieved by developing new formulations of cationic liposomes, or of other composite nonviral vector systems.

Romano, page 30, last paragraph.

Interestingly, Scherman *et al.*, in their discussion of *in vivo* gene transfer by lipoplexes, state that although they previously presented the inflammatory and complement-activating properties of lipoplexes as a limitation to the use of cationic lipids in gene therapy, they now state that "one might rather consider these data as encouraging." Scherman, page 482, last two paragraphs.

The Examiner has apparently grounded this aspect of the enablement rejection on the alleged low efficiency with which genetic vectors can be delivered *in vivo*. For example, the Examiner stated that:

> [t]he instant specification fails to teach one of skilled [sic] in the art how to overcome the unpredictability for *in vivo* vector targeting, such that an *efficient transfer and expression* of a nucleotide molecule encoding FPGS could be achieved in neoplastic cells *in vivo* through any route of delivery such that upon treatment with an antifolate drug, the drug is activated by the FPGS gene product to effect the killing of said neoplastic cells or enhance the cytotoxic sensitivity of the neoplastic cells.

Paper No. 13, page 7 (emphasis added).

To the contrary, Applicants respectfully submit that the specification does indeed provide guidance to those skilled in the art on improving vector targeting. Page 21, line 12, to page 22, line 11, of the specification teaches that the targeting specificity for FPGS gene delivery may be facilitated by targeted delivery or targeted expression ("transcriptional targeting"). The specification teaches and provides reference citations on the use of tumor-specific or tumor-selective DNA enhancer sequences to selectively activate expression of the transduced gene in the tumor cell at either the primary tumor site or its metastasis. Numerous examples of this approach are provided, including DNA enhancers

that have been derived from genes encoding tyrosinase (allowing for targeting to melanoma), ERBB2 (targeting to pancreatic cancer), carcinoembryonic antigen (targeting to lung and gastrointestinal malignancies, including colon, pancreatic and gastric cancer), DF3/MUC1 (targeting to breast cancer), and alpha-fetoprotein (targeting to hepatoma). The specification also teaches that the use of synthetic gene regulation systems, which allow for transcriptional control and other forms of regulated expression of the FPGS gene, may also be used. Finally, the specification teaches the use of DNA regulatory elements that are controlled by tumor-specific conditions and factors. This guidance on ways to improve vector targeting may be applied to virtually any gene delivery system.

The currently claimed methods are directed to "A method of enhancing the cytotoxic sensitivity of neoplastic cells to an antifolate drug, said method comprising: (a) delivering into said neoplastic cells a vector, said vector comprising a DNA sequence encoding folylpolyglutamyl synthetase (FPGS), operably linked to a promoter, wherein said FPGS is expressed in said neoplastic cells at a level higher than the endogenous FPGS level of said neoplastic cells; (b) treating the neoplastic cells in step (a) with an antifolate drug that is polyglutamated by said FPGS; and (c) enhancing the cytotoxic sensitivity of said neoplastic cells to said antifolate drug." Applicants have added new claims 33 and 34 to recite that the vector is delivered directly to the neoplastic cells (claim 33) and specifically by direct inoculation (claim 34).

Applicants' claims do not require that the vector carrying the FPGS gene be delivered into neoplastic cells with any particular minimum level of efficiency, so long as the cytotoxic sensitivity of the neoplastic cells are enhanced following antifolate treatment.

Applicants respectfully submit that one skilled in the art would be able to ascertain this by routine experimentation. *See, e.g.*, Example 1 of the present specification.

The above notwithstanding, it was recognized in the art at the time of Applicants' application, that a major advantage associated with gene delivery systems utilizing so called "suicide genes," such as FPGS, is that only a low level of gene delivery is required to exert anti-neoplastic effects.

> Suicide genes are used in a significant number of the cancer trials because they have a number of advantages. (1) Suicide genes and their prodrug are toxic to chemotherapy resistant tumors. (2) Only short-term gene expression is required. *(3) Only a fraction of the tumor cells within the tumor mass (>10%) needs to express the suicide gene to kill the entire tumor.* (4) Gene-modified tumor cells that die after exposure to the prodrug stimulate an immune response, which in some cases has been shown to be stronger than that to irradiated tumor cells. Taken together, these characteristics allow for cytoreduction of the tumor mass through "molecular surgery" by killing the tumor cells that have been genetically modified with the suicide gene.

Freeman, S.M., *et al.*, *Semin. Oncology 23*:31-45 (1996) (emphasis added, Table references omitted; already of record); *See also* Verma and Somia at 239 ("In the brain, however, gene transfer to *just a few hundred cells* could considerably benefit patients with neurological disease," emphasis added).

Finally, Applicants have provided evidence for a positive "bystander effect" in culture and *in vivo* using the presently claimed method. *See*, specification, pages 37-40. It is well-known in the gene therapy art that evidence of a positive bystander effect is important because of low transduction efficiencies of a particular gene. The "bystander effect" refers to the ability of transduced tumor cells to cause cytotoxicity in neighboring non-transduced tumor cells. It is well known that this is an important prerequisite for

success under real-world conditions, where the transfer of genes to cells within a tumor is substantially less than 100%.

Thus, as recognized by those skilled in the art, the successful application of the invention does not necessarily require high levels of efficiency of genetic vector delivery.

Applicants emphasize that the point of novelty of the presently claimed invention is that the expression of FPGS in neoplastic cells, when combined with antifolate drugs, will enhance the cytotoxic sensitivity of the neoplastic cells. The particular type of gene delivery system that can deliver the FPGS gene is broad (viral vectors, non-viral vectors (including cellular vectors), or a hybrid of the two; *see*, specification, pages 15-20), and will, of course vary, based on numerous factors including, the particular tumor type, the tumor location, the condition of the patient, and the capabilities and/or particular expertise of a given laboratory, to name a few. These factors, as well as others, will be taken into account by those skilled in the art when determining the best suited gene delivery system.

Finally, there are several examples in the literature at the time the present application was filed that demonstrate the effectiveness of *in vivo* genetic vector delivery by methods other than direct inoculation of tumors. For instance, Deonarian, discussed above, describes successful results involving the *in vivo* delivery of genetic vectors to liver cells. As another example, Lan *et al.*, *Cancer Res. 57*:4279-4284 (1997)(already of record), demonstrated the delivery of an i.p.-administered adenoviral vector to gastric carcinoma cells. In addition, Nakanishi, *Crit. Rev. Therapeu. Drug Carrier Systems 12*:263-310 (1995)(already of record), provides an overview of systems for gene transfer into tissue cells and summarizes several studies which illustrate the *in vivo* delivery of retroviral, adenoviral, adeno-

associated viral, and herpesviral vectors. Nakanishi further summarizes the state of *in vivo* gene transfer as of 1995:

> *In vivo* gene transfer is an approach to transfect tissue cells *in situ* by introducing gene transfer vectors through direct injection, through perfusion with catheters, or through an intravenous injection. This approach is more practical than *ex vivo* gene transfer and will become the major route for therapeutic gene transfer in the future. *In vivo* transfer may be applied to a wide variety of tissues and cells, and many vector systems other than retroviral vectors have been reported to be adopted for *in vivo* transfection.

Nakanishi at page 267. Finally, Roth *et al.*, *J Natl. Cancer Inst. 88*:21-39 (1997)(already of record) report that certain gene-based therapies for cancer, including drug sensitization with genes for prodrug delivery similar in nature with the presently claimed invention, have resulted in tumor regressions or killing cancer cells (see the entire article, particularly pages 21-24, as well as the Appendix Table 1).

Thus, the state of the art as of the effective filing date of the present application clearly indicates that the *in vivo* delivery of genetic vectors to target cells by methods besides direct inoculation is feasible and has important clinical applications in the field of gene therapy.

Another issue raised by the Examiner concerns host immunological responses to the delivered vectors, as a factor limiting effective gene therapy *in vivo*. As discussed in the specification at page 42, lines 2-13, one of the advantages of FPGS gene therapy is that the gene product expressed by the tumor cells (FPGS) is not a foreign enzyme, and therefore should not elicit an immune response, as might be the case with expression of a foreign "suicide" gene (*i.e.*, herpes simplex virus-thymidine kinase, bacterial cytosine deaminase),

which may generate an immune response before the tumor cells are able to mediate an effective bystander effect.

To summarize, the references cited by the Examiner in support of the rejection merely indicate that, in certain contexts and using certain approaches, technical difficulties may exist that impede the delivery of genetic vectors with optimum efficiency. Applicants' claims, however, do not specify any minimum level of genetic vector transfer efficiency, so long as cytotoxic sensitivity to an antifolate drug is enhanced. The Examiner has not established that the *in vivo* delivery of genetic vectors by methods other than direct inoculation is of such low efficiency so as to be regarded as inoperative. In fact, at the time the present application was filed, the scientific literature (including even the references cited by the Examiner) was replete with examples of successful *in vivo* genetic vector delivery using methods other than direct inoculation; that is, it would not require undue experimentation for a skilled artisan to practice the full scope of Applicants' invention, including the delivery of viral and non-viral based genetic vectors by *in vivo* methods. The Examiner has not provided acceptable objective evidence that this is not the case, and therefore has not met his burden for *prima facie* non-enablement under *In re Marzocchi, supra*.

Therefore, in addition to *in vitro* genetic vector delivery and the delivery of genetic vectors by direct inoculation, Applicants' claims are fully enabled with respect to *in vivo* genetic vector delivery methods.

Prokaryotic Vectors

The Examiner also asserts that with respect to the elected species of a prokaryotic

vector, the claims are not necessarily limited to a live bacterial vector for gene delivery to neoplastic cells and that "the claims encompass any prokaryotic vector." *See*, Paper No. 13, pages 9-10. The Examiner concludes that the specification is not enabled for such a broadly claimed invention because it does not provide any guidance for a skilled artisan on how to make and use any prokaryotic vector (both pathogenic and non-pathogenic) to be functional for expressing a transgene in a mammalian cell such as a neoplastic cell.

Applicants respectfully submit that the present specification teaches that prokaryotic vectors, such as, for example, the tumor targeted bacterial vectors of Pawelek *et al., Cancer Res. 57*:4537-4544 (1997), can be used to deliver the FPGS gene to neoplastic cells. *See*, specification, page 19, line 27, to page 20, line 1. Pawelek used tumor-targeting *Salmonella* to deliver the HSV thymidine kinase gene to mice with melanoma, and observed suppression of tumor growth following treatment with ganciclovir. A more recent paper by the same group, Bermudes *et al.*, *Adv. Exp. Med. Bio. 465*: 57-63 (2000), characterizes tumor-targeted *Salmonella* as highly selective delivery vectors. Bermudes *et al.* also discuss the success of genetically engineered *Salmonella* in the expression and delivery of prodrug-converting enzymes. Bermudes *et al.*, pages 61-62. A copy of this paper is attached as Exhibit A.

Applicants enclose herewith an additional reference that supports the enablement of prokaryotic vectors in the claimed method. Lemmon M.J., *et al.*, *Gene Ther :4*:791-796 (August 1997) reports on the use of anaerobic bacteria as a *systemic* gene delivery system that is controlled by the tumor microenvironment. Genetically engineered *Clostridia* are used as tumor-specific vectors for the delivery of antitumor genes. Lemmon M.J., *et al.* introduced into *C. beijerinckii,* the gene for an *E. coli* nitroreductase (known to activate the nontoxic prodrug CB 1954 to a toxic anticancer drug). Nitroreductase produced by the

Clostridia enhanced the killing of tumor cells *in vitro* by CB 1954, by a factor of 22. To demonstrate the specificity of this approach for tumor targeting, Lemmon M.J., *et al.* intravenously injected the inactive spore form of *C. beijerinckii*, which upon transition to a reproductive state will express the *E. coli* nitroreductase gene. Nitroreductase activity was detectable in 10 of 10 tumors during the first 5 days after intravenous injection of inactive clostridial spores, indicating a rapid transition from spore to reproductive state. Tumors harboring clostridial spores, which did not possess the *E. coli* nitroreductase gene, were devoid of nitroreductase activity. Most importantly, *E. coli* nitroreductase protein was not found in a large survey of normal mouse tissues following intravenous injection of nitroreductase containing clostridia. The authors conclude that obligate anaerobic bacteria such as *Clostridia* can be utilized as highly specific gene delivery vectors for cancer therapy. A copy of this paper is attached as Exhibit B.

Applicants respectfully submit that the Examiner has not cited any objective evidence or provided sound scientific reasoning to doubt the enablement of prokaryotic vectors as a means of gene delivery.

Replication Competent Viral Vectors and/or Pathogenic Live Prokaryotic Vectors

The Examiner also asserts that with respect to an embodiment of the claims encompassing the use of replication competent viral vectors and/or pathogenic live prokaryotic vectors for gene delivery into neoplastic cells via a systemic route, "[n]either the instant specification nor the prior art at the effective filing date of the present application teaches the use of replication competent viral vectors such as retrovirus, adenovirus or lentivirus (HIV-1 and HIV-2) or pathogenic live prokaryotic vectors for achieving the

contemplated therapeutic results." *See,* Paper No. 13, page 10. The Examiner further suggests that the use of replication competent viral vectors or pathogenic live prokaryotic vectors would impede the practice of the claimed methods:

> It is unclear whether the treated individual having neoplastic cells would succumb to the cytotoxic effects of replication competent viral vectors or pathogenic prokaryotes prior to any therapeutic effects contemplated by Applicants could be attained. Pawelek *et al.* noted that when wild-type *Salmonella* were introduced into melanoma-bearing mice, the bacteria [were] found within the tumor at levels exceeding 10^9 per gram, although as pathogens, they caused the death of the mice. Hollon, T. (*Nature Med. 6* (1): 6, 2000) also reported the first gene therapy death resulting from the utilization of replication-deficient adenovirus in a patient, let alone the use of replication competent HIV or other retroviral viruses.

Paper No. 13, page 10.

The Examiner concludes that with the lack of guidance in the specification for this embodiment, it would have required undue experimentation for a skilled artisan to make and use the full scope of the methods as claimed.

In essence, the Examiner has based this aspect of the enablement rejection on the presence of alleged inoperative embodiments, *i.e.*, methods for killing neoplastic cells using replication competent viral vectors or pathogenic live prokaryotic vectors. As an initial matter, Applicants submit that the Examiner has not provided sufficient evidence to support his conclusions.

Regarding the statement in the Pawelek Abstract that the melanoma-bearing mice were killed following the introduction of wild-type *Salmonella*, Applicants direct the Examiner's attention to the following sentence in the Abstract, which states "However, when

attenuated, hyperinvasive autotrophic mutants were used, the tumor-targeting and amplification phenomena were retained, whereas their pathogenicity was limited." The Abstract concludes that "attenuated *Salmonella* would be useful both for inherent antitumor activity and delivery of therapeutic proteins to cancer cells *in vivo*." Pawelek Abstract.

Regarding the Examiner's citation of the first gene therapy death in the Hollon, T. document (where a replication-deficient adenovirus vector was used) , Applicants submit that a single death in a clinical trial for treatment of a totally different disease (partial ornithine transcarbamylase deficiency) than currently claimed, does not support the non-enablement for any and all replication-competent viral vectors. Applicants submit herein a review article by Galanis and Vile, *Crit Rev Oncol Hematol 38*:177-192 (2001), which focuses on the use of targeting and replication competent vectors to overcome challenges in gene therapy, such as low efficiency of gene transfer and target selectivity. A copy of this reference is attached as Exhibit C.

Further, the Examiner contends that neither the specification nor the prior art teach the use of replication competent viral vectors. Applicants respectfully disagree. Page 19, lines 13-21, of the specification states:

> Preferably, the viral genomes of the viral vectors used in the invention should be modified to remove or limit their ability to replicate, however, *replication conditional viruses will also be useful in the present invention, as will replicating vectors that are capable of targeting certain cells. See, e.g.*, Zhang, J., *et al., Cancer Metastasis Rev. 15*:385-401 (1996). Chase, M., *et al. (Nature Biotechnol. 16*:444-448 (1998)) used a herpes virus with an inactivated viral ribonucleotide reductase gene that selectively delivered P450 2B1 to tumor cells that overexpress the mammalian ribonucleotide reductase enzyme, which is required for this modified virus to replicate. (Emphasis added).

Thus, contrary to the Examiner's assertion, the present specification does indeed provide guidance for using a viral vector that is not necessarily replication-*defective*. Further, Applicants direct the Examiner's attention to the following U.S. Patents: 5, 585,096 (1996) and 6,106,826 (2000), which are directed to viral vectors that are replication competent, yet avirulent, that find use in mammalian gene therapy.

The above notwithstanding, Applicants note that the possible presence of inoperative embodiments within the scope of a claim does not necessarily render a claim non-enabled. *See, Atlas Powder Co. v. E.I. du Pont de Nemours & Co.*, 224 U.S.P.Q. 409, 414 (Fed. Cir. 1984). The standard is whether a skilled artisan could determine which embodiments of the claimed invention would be inoperative or operative with expenditure of no more effort than is normally required in the art. *See id.*; *see also In re Angstadt*, 190 U.S.P.Q. 214, 218 (CCPA 1976). Applicants submit that one skilled in the art of vector biology and cancer gene therapy would know, based on the guidance in the specification, the art, and their background knowledge, which viral or non-viral vectors would be suitable for use in the currently claimed method. Applicants assert that it would take no more than routine experimentation on the part of the skilled artisan to ascertain which vector systems would and would not be appropriate for use within the scope of the claimed invention. The state of the art as of the effective filing date of the present application was such that the various classes and variants of viral vectors (and other vehicles for the delivery of genetic material to cells) were well defined and understood. *See, e.g.,* Nakanishi, *supra*, for an overview of various methods for gene transfer into cells.

Applicants submit that it is unnecessary for Applicants to exclude replication competent vectors from the scope of the present claims, as several types of these vectors

(*e.g.*, replicating vectors that are capable of targeting certain cell types or replication conditional/replication provisional vectors), clearly find use in the practice of the claimed methods.

Mammalian Artificial Chromosomes

The Examiner asserts that with respect to claims specifically encompassing the use of mammalian artificial chromosomes as a non-viral gene delivery of the FPGS gene for enhancing the cytotoxic sensitivity of neoplastic cells to an antifolate drug, "the instant specification fails to provide any specific teachings regarding to [sic] the making or using of any mammalian artificial chromosome in a method as claimed." Paper No. 13, page 11.

Applicants note that they are not limited to the confines of the specification to provide the necessary information to enable the invention. *See, In re Howarth*, 210 U.S.P.Q. 689, 692 (CCPA 1981). It is well-established that an Applicant need not supply information that is well-known in the art. *Id.*, 210 U.S.P.Q. at 692; *see also In re Brebner*, 173 U.S.P.Q. 169 (C.C.P.A. 1972) (finding a disclosure enabling because the procedure for making the starting material, although not disclosed, would have been known to one of ordinary skill in the art as evidenced by a Canadian patent).

Applicants assert that the construction and use of mammalian artificial chromosomes was well known in the art as of the filing date of the present application. *See e.g.*, Specification at page 20, line 9-10 (citing Ascenzioni, *et al., Cancer Lett. 118*:135-142 (1997), which provides an overview of the technology regarding mammalian artificial chromosomes). Thus, it would have been unnecessary, indeed improper, for Applicants to

provide detailed instructions regarding the construction of mammalian artificial chromosomes.

The Examiner cites a passage from Calos, *Trends Genet. 12*:463-466 (1996), that allegedly lends support to the assertion that " . . . in the absence of any *in vivo* example demonstrating an effective use of an artificial chromosome vector comprising a nucleotide sequence encoding FPGS for enhancing the cytotoxic sensitivity to an antifolate drug or killing neoplastic cells *in vivo*, it would have required undue experimentation for a skilled artisan to make and use this particular embodiment of the presently claimed invention." *See,* Paper No. 13, page 13. The passage cited by the Examiner is as follows:

> A vector of this size is far beyond the size of vectors in current use for gene therapy and poses problems of major dimensions, particularly for the manufacture and delivery of ~~vector DNA. Therefore, while construction of artificial~~ chromosome vectors has not yet been realized, once it is, a series of challenging technical barriers will have to be surmounted before such molecules could reasonably be used as gene therapy vectors. (Emphasis in original).

Calos at page 464, right column.

Applicants note that, in the above-quoted passage, the author is referring to artificial chromosomes created by a very specific strategy, namely, the progressive reduction of existing chromosomes using telomere-mediated fragmentation. *See id.* Moreover, when read in its full context, the author's statement amounts to, at most, an educated guess as to the minimum size that might be achieved for an artificial chromosome based on the *prediction* that "[t]he final size [of an artificial chromosome produced by telomere-mediated fragmentation] will *probably* be driven primarily by the minimal size of a stable centromere, *which appears* to be about 1 Mb." *See id,* (emphasis added).

Therefore, the only conclusion that can be reasonably drawn from the statements in Calos is that technical difficulties *may* be encountered in the production of artificial chromosomes when they are generated by the progressive reduction of existing chromosomes using telomere-mediated fragmentation, *assuming* that the minimal size of a stable centromere, is about 1 Mb. Applicants maintain that such tentative conclusions do not suggest that the practice of the claimed invention insofar as it encompasses the use of mammalian artificial chromosomes would require any more than routine experimentation.

In their prior Reply, Applicants provided copies of two Abstracts: (1) Huxley, C., "Mammalian Artificial Chromosomes; a New Tool for Gene Therapy," *Gene Therapy 1*:7-12 (1994); and (2) Vos, J.M., "Mammalian Artificial Chromosomes as Tools for Gene Therapy", *Curr Opin Genet Dev 8*:351-9 (1998), which were also available to those skilled in the art as of Applicants' filing date. Clearly, those documents provide guidance to those skilled in the art on the use of Mammalian Artificial Chromosomes in the context of gene therapy.

Even if the Examiner's assessment of mammalian artificial chromosomes was correct, however, (a proposition which Applicants explicitly traverse), the rejection would amount to no more than a rejection on the basis of an alleged inoperative embodiment. As stated above, the presence of inoperative embodiments within the scope of a claim does not necessarily render a claim non-enabled. *See, Atlas Powder Co.*, 224 U.S.P.Q. at 414. A rejection for lack of enablement on the basis of an inoperative embodiment is only proper when it is shown that a skilled artisan would require more than routine experimentation to distinguish the inoperative from the operative embodiments. *See id.*

With respect to the present claims, the Examiner has provided no evidence or arguments to suggest that the reasonable practice of the claimed invention would be substantially impeded due to the presence of mammalian artificial chromosomes within the scope of the claims. Nor has the Examiner set forth any objective evidence that it would require undue experimentation to distinguish the operative from the supposed inoperative embodiments of the claimed invention. Accordingly, the presence of mammalian artificial chromosomes within the scope of the present claims does not provide a permissible basis of rejection under 35 U.S.C. § 112, first paragraph.

Summary

Applicants assert that the Examiner, in providing the grounds of rejection discussed above, has not met his initial burden of establishing a reasonable basis to question the enablement provided for the claimed invention. *See, In re Wright*, 27 U.S.P.Q.2d 1510, 1513 (Fed. Cir. 1993); *In re Marzocchi*, 169 U.S.P.Q. 367, 369 (CCPA 1971). Further, the possible presence of inoperative embodiments within the scope of a claim does not necessarily render a claim non-enabled. *See, Atlas Powder Co. v. E.I. du Pont de Nemours & Co.*, 224 U.S.P.Q. 409, 414 (Fed. Cir. 1984). The standard for enablement is whether a skilled artisan could determine which embodiments of the claimed invention would be inoperative or operative with expenditure of no more effort than is normally required in the art. *See id.*

Accordingly, since a *prima facie* case of non-enablement has not been made, Applicants respectfully request that all of the grounds of rejection under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

Rejections Under 35 U.S.C. § 112, Second Paragraph

The Examiner rejects claims 14-32 under 35 U.S.C. § 112, second paragraph, because, according to the Examiner, these claims are indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. *See,* Paper No. 13, pages 19-20.

Claims 14, 32, and Claims Dependent on Claim 14

The Examiner asserts that in claims 14, 32 and the claims dependent on claim 14, the phrase "treating said neoplastic cells" in step (b) is unclear. *See,* Paper No. 13, page 19.

Although Applicants disagree with the grounds of this rejection, and believe that one skilled in the art would surely understand that the neoplastic cells in step (b) are the ones that were treated with the FPGS-carrying vector, solely to expedite prosecution, the amended claims now state that the neoplastic cells are the ones from step (a).

In addition, Applicants have amended step (c) of claims 14 and 32 to change "neoplastic cell" to "neoplastic cells," to provide a more accurate antecedent basis for the term. Applicants thank the Examiner for noticing this typographical error.

Thus, this rejection under 35 U.S.C. § 112, second paragraph, has been overcome and should be withdrawn.

Claim 28

The Examiner asserts that, in claim 28, the phrase "said vector for gene delivery" lacks sufficient antecedent basis in claim 14. *See*, Paper No. 13, page 20.

This rejection is rendered moot by the amendment to claim 28. Applicants note that a similar amendment was made to other claims containing the same language.

In addition, the Examiner stated that it was unclear, and requested clarification, on how a vector comprising a nucleotide molecule encoding FPGS could be a cationic peptide, a starburst polyamidoamine dentrimer, a cationic liposome, or a fusogenic liposome.

Applicants respectfully submit that one skilled in the art of non-viral vector biology and gene therapy would surely understand the means by which cationic or fusogenic liposomal gene delivery or peptide-mediated gene delivery methods involve the formation of a lipid- or protein- complex with DNA in order to deliver the DNA to a target cell. *See, e.g.*, Christiano R.J., *"Targeted Non-Viral Gene Delivery for Cancer Gene Therapy," Front. Biosci. 3*: D1161-1170 (1998). A copy of this reference is attached as Exhibit D.

This rejection under 35 U.S.C. § 112, second paragraph, has been overcome and should be withdrawn.

Claims 20, 21 and Their Dependent Claims

The Examiner asserts that, in claims 20, 21, and their dependent claims, the phrase "said chemotherapeutic agent" lacks antecedent basis. *See*, Paper No. 13, page 20.

This rejection is rendered moot by the amendment of claims 20 and 21 to recite the phrase "antifolate drug" in lieu of "chemotherapeutic agent." This rejection under 35 U.S.C. § 112, second paragraph, has been overcome and should be withdrawn.

Rejections Under 35 U.S.C. § 103

The Examiner rejects claims 14-19, 22, 25-28, 31 and 32 under 35 U.S.C. § 103(a), because, according to the Examiner, these claims are unpatentable over Moscow *et al.*, U.S. Patent 5,763,216 ("the Moscow patent"), in view of Roy *et al.*, *J. Biol. Chem. 272*: 6903-6908 (1997) ("Roy"), Kim *et al.*, *J. Biol. Chem. 268*:21680-21685 (1993) ("Kim"), and Garrow *et al.*, *Proc. Nat'l. Acad. Sci. 89*:9151-9155 (1992) ("Garrow"). *See,* Paper No. 13, pages 20-25.

The Examiner states:

> It would have been obvious and within the scope of skills for an ordinary skilled artisan to modify the method of Moscow *et al.* by direct delivery of a non-viral (plasmid) or viral vector comprising a DNA sequence encoding human FPGS into neoplastic cells *in vivo* that have acquired resistance to methotrexate and other classical folate analogues in order to reverse the resistance of MTX or other antifolate drugs in these neoplastic cells, so that to enhance the efficacy of conventional anti-folate drug therapy in light of the teachings of Roy *et al.*, Kim *et al.*, and Garrow *et al.* It is noted that as defined by the present application, a neoplastic cell is a cell whose normal growth control mechanism is disrupted thereby providing the potential for uncontrolled proliferation (citations omitted). As such, tumor cells resistant to MTX or other antifolate drugs would be encompassed within the scope of neoplastic cells of the instant invention. Furthermore, by reversing the resistance to MTX and other antifolate drugs in the tumor cells, the cytotoxic sensitivity of the tumor cells to an antifolate drug is in effects [sic] enhanced.

Paper No. 13, pages 23-24.

The Examiner also states:

> One of ordinary [skill] in the art would have been motivated to carry out the above modification because Moscow *et al.*, Roy *et al.* and Kim *et al.* recognize that

> decreased folylpolyglutamate synthetase is a factor contributing to the resistance of tumor cells to methotrexate or other antifolate drug treatment, and by increasing the exogenous expression of FPGS in MTX or other antifolate resistant tumor cells, the sensitivity to antifolate drugs of the treated tumor cells would be enhanced and thereby enhancing the efficacy of traditional antifolate chemotherapy. (Emphasis in original).

Paper No. 13, page 24, lines 7-14.

Finally, the Examiner states:

> One of an ordinary skilled artisan would have a reasonable expectation of success because Kim *et al.* clearly teach that lowered FPGS activity and decreased polyglutamylation of antifolates are thought to be general mechanisms by which cancer cells become resistant to a wide range of antifolates, and that FPGS-deficient mutant Chinese hamster ovary (CHO AUXB1) cells expressing high levels of human ... FPGS are more sensitive to the cytotoxicity of MTX compared to cells expressing lower levels of human FPGS (see Table III, page 21682). Furthermore, Roy *et al.*clearly show that L1210 tumor cells resistant to methotrexate or edatrexate have lowered FPGS activity. Therefore, the claimed invention as a whole was *prima facie* obvious in the absence of evidence to the contrary. (emphasis in original).

Paper No. 13, pages 24-25. Applicants respectfully traverse the rejection.

The Moscow patent relates to the human reduced folate carrier (RFC) gene, expression vectors comprising the RFC gene, as well as the use of such vectors to restore methotrexate (MTX) sensitivity to *MTX-resistant*, transport deficient, cancer cells. As discussed in the "Background of the Invention" section of the Moscow patent, RFC is involved in one of two pathways for folate transport across cell membranes as well as the uptake of folate by animal cells. Accordingly, the RFC system is involved in facilitating MTX (folate antagonist) uptake, and although other genes besides RFC may be involved

in the development of MTX-resistance (including decreased FPGS), "decreased MTX uptake is the principal characteristic in many MTX-resistant cell-lines." Moscow Patent, col. 1, 2nd paragraph. Thus, clearly, the Moscow patent is concerned with: (1) the RFC gene, and not the FPGS gene; and (2) the restoration of MTX sensitivity to MTX-*resistant*, transport deficient cancer cells, and not the enhancement of cytotoxic sensitivity when FPGS is expressed in neoplastic cells at a level higher than the endogenous FPGS level of said neoplastic cells.

As discussed below, Roy, Kim, and Garrow do not remedy the fundamental deficiencies in the Moscow patent.

Roy teaches that L1210 cell variants which express either decreased or increased levels of FPGS (compared with parental lines) display corresponding differences in resistance to folate analogues. According to the Examiner, Roy teaches that L1210 tumor cells *resistant to MTX* have a decrease in the rate of FPGS mRNA transcript formation, resulting in lower FPGS activity (page 6907, col.2, first full paragraph).

Kim teaches that mutant CHO cells, lacking FPGS activity, exhibit increased sensitivity to pulses of MTX in cell culture after being transfected with an FPGS expression cassette. Thus, Kim teaches that FPGS cDNA transfection restored cytotoxic sensitivity of FPGS-deficient CHO cells to methotrexate. Kim does not teach the transformation or transfection of neoplastic cells. In addition, Kim does not teach the transformation or transfection of cells which have some endogenous FPGS activity (like neoplastic cells), nor show that such transformation or transfection with an FPGS gene can enhance the neoplastic cell's cytotoxic sensitivity to an anti-folate drug.

Garrow teaches the cloning of a human FPGS. Garrow teaches that transfecting the cloned FPGS into mutant CHO cells lacking FPGS activity restored the ability of the transfected mutant cells to grow in culture in the absence of purines and thymidine. Garrow does not teach the delivery of a vector comprising a nucleotide molecule that encodes an FPGS into *neoplastic cells*, nor does Garrow teach the treatment of cells expressing FPGS with an antifolate drug. Further, Garrow doesn't teach or suggest that transformation or transfection of neoplastic cells with an FPGS gene can enhance the neoplastic cell's cytotoxic sensitivity to an anti-folate drug.

Rejection of claimed subject matter as obvious under 35 U.S.C. § 103 in view of a combination of references requires (1) consideration of whether prior art would have suggested to those of ordinary skill in the art that they should make the claimed composition or carry out the claimed process, and (2) whether the prior art would also have revealed that such a person would have reasonable expectation of success; both suggestion and reasonable expectation of success must be found in the prior art, not in Applicant's disclosure. *See, In re Vaeck*, 20 U.S.P.Q.2d 1438, 1442 (Fed. Cir. 1991). Further, all claim limitations must be taught or suggested by the prior art. *In re Royka*, 180 U.S.P.Q. 580 (CCPA 1974).

Applicants assert that the Examiner has not established a *prima facie* case of obviousness because he has not pointed to anything, in the cited references or in the body of knowledge generally possessed by those skilled in the art, that would suggest the modification or combination of the references necessary to arrive at Applicants' claimed invention. Although it might have been obvious for the skilled artisan to try and see if cytotoxic sensitivity of neoplastic cells to anti-folate drugs could be enhanced by introducing an FPGS gene, such does not give rise to a *prima facie* case of obviousness. *See, In re*

Geiger, 2 U.S.P.Q.2d 1276 (Fed. Cir. 1987) and *In re Fine*, 5 U.S.P.Q.2d 1596 (Fed. Cir. 1988).

Applicants presently pending claims are not directed to restoring cytotoxic sensitivity to neoplastic cells that are *resistant to methotrexate* or other folate analogues. The present claims are directed to: "A method of enhancing the cytotoxic sensitivity of neoplastic cells to an antifolate drug, said method comprising:

(a) delivering into said neoplastic cells a vector, said vector comprising a DNA sequence encoding folylpolyglutamyl synthetase (FPGS), operably linked to a promoter, *wherein said FPGS is expressed in said neoplastic cells at a level higher than the endogenous FPGS level of said neoplastic cells*;

(b) treating the neoplastic cells in step (a) with an antifolate drug that is polyglutamated by said FPGS; and

(c) *enhancing the cytotoxic sensitivity of said neoplastic cell to said antifolate drug."* (Emphasis added).

In rationalizing the rejection, the Examiner has relied on the theory in Kim that "[l]owered FPGS activity may be a general mechanism by which cells can become resistant to a wide range of antifolates." This theory, however, does not provide the requisite motivation to modify or combine the cited references, and arrive at the presently claimed invention.

Applicants note that the claims are not dependent on any particular mechanism of action, nor do they necessarily require that the neoplastic cells be MTX resistant. The point of novelty of the currently claimed invention is the teaching and demonstration that, in the

context of vector-mediated gene therapy, the elevation of FPGS activity beyond the endogenous level characteristic of a particular tumor cell, will augment their cytotoxic sensitivity.

As discussed above, Kim shows that vector-mediated transfection of FPGS cDNA can restore FPGS activity and reintroduce cytotoxic sensitivity into variant CHO cells that express *no endogenous* FPGS activity. In contrast, the claimed invention recites that the FPGS is transferred to neoplastic cells which have *some* endogenous FPGS activity. Applicants have shown that elevation of FPGS activity via vector-mediated gene therapy, *beyond the endogenous level characteristic of most tumor cells*, will augment their cytotoxic sensitivity. That is, the issue of whether tumor cells, already expressing FPGS, can be imbued with enhanced antifolate sensitivity after FPGS gene delivery has not been previously addressed by any of the cited art, taken alone or in combination. The limitations of the claimed method have not been met.

Applicants contend that the Examiner has not provided a sufficient explanation as to why a person skilled in the art would have been motivated to modify the teachings of Kim or Garrow such that the cloned FPGS gene is delivered, not to a mutant Chinese hamster ovary cell, but to a *neoplastic cell.*

Since there is no motivation to modify or combine the cited references to arrive at Applicants' claimed invention, and the Examiner has failed to point to any such motivation, a *prima facie* case of obviousness has not been established. Accordingly, Applicants respectfully request that the rejection of the claims under 35 U.S.C. § 103(a) be reconsidered and withdrawn.

Next, the Examiner rejects claims 14 and 28-29 under 35 U.S.C. § 103(a), because, according to the Examiner, these claims are unpatentable over the Moscow patent, in view of Roy, Kim, and Garrow, as applied to claims 14-19, 22, 25-28, 31 and 32 above, and further in view of Pawelek *et al.*, *Cancer Research 57*:4537-4544 (1997)("Pawelek"). *See,* Paper No. 13, pages 25-26. Applicants respectfully traverse the rejection.

The teachings of all references, except Pawelek, are discussed and distinguished above. Pawelek teaches the use of an attenuated *Salmonella* as an anticancer vector for gene delivery into tumor cells. The Examiner contends that it would have been obvious for a skilled artisan to modify the combined teachings of Moscow, Roy, Kim, and Garrow for delivering a DNA sequence encoding human FPGS into tumor cells resistant to methotrexate and other folate analogues by using an attenuated *Salmonella* as an anticancer gene delivery vector, as taught by Pawelek.

Applicants respectfully submit that Pawelek does not remedy any of the fundamental defects of the prior rejection, see *supra*. Accordingly, this rejection is improper and should be withdrawn.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided. Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Karen R. Markowicz

Karen R. Markowicz
Agent for Applicants
Registration No. 36,351

Date: April 4, 2003

1100 New York Avenue, N.W.
Washington, D.C. 20005-3934
(202) 371-2600

::ODMA\MHODMA\SKGF_DC1;119340;1

Version with markings to show changes made

Claims 33-36 have been added.

14. (Once amended) A method of enhancing the cytotoxic sensitivity of neoplastic cells to an antifolate drug, said method comprising:

(a) delivering into said neoplastic cells a vector, said vector comprising a [nucleotide molecule] DNA sequence encoding folylpolyglutamyl synthetase (FPGS), operably linked to a promoter, wherein said FPGS is expressed in said neoplastic cells at a level higher than the endogenous FPGS level of said neoplastic cells;

(b) treating [said] the neoplastic cells in step (a) with an antifolate drug that is polyglutamated by said FPGS; and

(c) enhancing the cytotoxic sensitivity of said neoplastic [cell] cells to said antifolate drug.

20. (Once amended) The method of claim 16, wherein said [chemotherapeutic agent] antifolate drug is methotrexate.

21. (Once amended) The method of claim 16, wherein said [chemotherapeutic agent] antifolate drug is edatrexate.

22. (Once amended) The method of claim 14, wherein said vector [for gene delivery] is a viral vector.

23. (Once amended) The method of claim 20, wherein said vector [for gene delivery] is a viral vector.

24. (Once amended) The method of claim 21, wherein said vector [for gene delivery] is a viral vector.

27. (Once amended) The method of claim 14, wherein said vector [for gene delivery] is non-viral.

28. (Once amended) The method of claim 14, wherein said vector [for gene delivery] is a prokaryotic vector, a cationic liposome, a fusogenic liposome, a DNA-adenovirus conjugate, a DNA-protein complex, a non-viral T7 autogene vector, a starburst polyamidoamine dendrimer, a cationic peptide, or a mammalian artificial chromosome [, an endothelial cell, or a macrophage].

29. (Once amended) The method of claim 27, wherein said vector [for gene delivery] is a prokaryotic vector.

30. (Once amended) The method of claim 14, wherein [the] said vector [for gene delivery] is delivered into said neoplastic cells by direct injection of nucleic acid, particle-mediated gene transfer, or receptor-mediated gene transfer.

32. (Once amended) A method of enhancing the cytotoxic sensitivity of neoplastic cells to methotrexate or edatrexate, said method comprising:

(a) delivering into said neoplastic cells a vector, said vector comprising a [nucleotide molecule] DNA sequence encoding folylpolyglutamyl synthetase (FPGS), operably linked to a promoter, wherein said FPGS is expressed in said neoplastic cells at a level higher than the endogenous FPGS level of said neoplastic cells;

(b) treating [said] the neoplastic cells of step (a) with methotrexate or edatrexate; and

(c) enhancing the cytotoxic sensitivity of said neoplastic [cell] cells to said methotrexate or edatrexate.

EXHIBIT A

6

TUMOR-TARGETED *SALMONELLA*

Highly Selective Delivery Vectors

David Bermudes[1]*, Brooks Low[2], and John Pawelek[3]

[1]Vion Pharmaceuticals, Inc.
4 Science Park, New Haven
Connecticut 06511 USA and
Yale University
School of Medicine
Departments of Therapeutic Radiology[2] and Dermatology[3]
333 Cedar Street, New Haven
Connecticut 06520 USA

1. BACKGROUND

The development of novel cancer therapies presents a formidable challenge to find means to selectively target and eradicate neoplastic cells while sparing healthy tissue. Cancerous cells arising from normal cells in the body have few and often subtle differences from their normal counterparts. Vasculature supplying the tumor is generally irregular and positive pressure within the tumor combines to create a physical barrier to penetration, often reducing the effectiveness of therapeutic agents (Boucher *et al.*, 1996; Jain, 1994). Thus the mandate for new therapeutic agents is not only to exploit differences that tumors may exhibit whereby the agent used is more toxic to cancerous cells than to normal cells, but to overcome the barriers that limit their delivery to the tumor.

One approach has been to develop methods to selectively bind to tumor cells using antibodies. Highly specific monoclonal antibodies directed to tumor-associated antigens such as Lewis y or HER2/neu, have the ability to be administered sysemically and to preferentially accumulate at the site of the tumor cells to which they bind. The anti-cancer activity of these antibodies has been enhanced in several ways. Such antibodies have been used to target cells overexpressing certain antigens and carry enzymes that activate non-toxic prodrug forms of chemotherapeutic agents, inducing selective toxicity at the site of

* To whom correspondence should be sent: DBermude@Vionpharm.com, Tel: (203) 498-4210 ×304, Fax: (203) 498-4211

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accumulation. These antibodies have also been directly coupled to toxic agents, or used in combination with them, including small molecule chemotherapeutics such as doxirubicin (Trail *et al.*, 1993; Baselga *et al.*, 1998) or protein toxins such as pseudomonas endotoxin (Friedman *et al.*, 1993; Siegall, 1995).

Yet while these or similar agents hold promise for providing selective delivery to tumors, they are subject to the same limitations posed by the fundamental barriers encountered by other agents requiring both the vasculature and diffusion to accomplish delivery into poorly vascularized tumors having positive pressure. Thus, antibody agents would be expected to be limited to the periphery of tumors and the selective accumulation in tumors predicted to be relatively low (Jain, 1994).

Gene therapy agents directed towards cancer are based on the principle of altering the cancer cells by introduction of genetic material in the form of modified viruses, plasmids, ribozymes, and other nucleic acid-based vectors. Specificity for cancerous cells is achieved by several means, including dependency on p53 mutations (Bischoff *et al.*, 1996), tumor specific promoters (e.g., Gotoh *et al.*, 1998), or direct intratumoral injection. By and large, these agents lack ability as systemically delivery vehicles, limiting their usage to local-regional disease.

Liposomes have also been developed for use as gene therapy delivery vehicles. Modification of the spheroids with antibodies provides some selectivity towards the target tissue (Allen *et al.*, 1995). These agents can be administered systemically but encounter difficulty in achieving the level of delivery that enables them to be effective.

Overall, current approaches to tumor-targeted therapy remains promising, yet most of the approaches require further development.

2. TUMOR-TARGETED BACTERIA

Systemically administered bacteria are a little-studied approach that offers surprising advantages over other systems. Clostridia were long ago tested as anti-tumor agents (Parker *et al.*, 1947; Möse and Möse, 1964; Carey *et al.*, 1967) and continue to be investigated (Fox *et al.*, 1996; Lemmon *et al.*, 1997). These bacteria, it was recognized, have a built-in specificity for tumors in that they are strict anaerobes, and can flourish within the anaerobic regions of large tumors. Smaller tumors, however, are not targeted. These bacteria multiply within the necrotic area, and achieve levels of 10^5–10^6/gm tumor tissue (Lemmon *et al.*, 1997).

We have investigated *Salmonella* as tumor-targeting agents (Pawelek *et al.* 1997). These bacteria are facultative anaerobes, growing well in oxygenated as well as hypoxic regions of the tumor. *Salmonella* are motile, and thereby overcome diffusion and pressure gradients that exist in tumors. They are also sensitive to a wide range of antibiotics, and can be engineered to exhibit many of the properties desired in an antitumor delivery vehicle.

Salmonella typhimurium is a common cause of food poisoning frequently contracted by eating undercooked chicken, eggs, and other foods. In humans, disease is usually self-limiting, confined to the lower intestines. Occasionally, the bacteria escape the confines of the gut into circulation, resulting in bacteremia and in some cases septic shock and death if left untreated. Mice are highly sensitive to *S. typhimurium*, and relocation of the bacteria from the lower intestines into circulation occurs at a high frequency similar to the etiology of thyphoid fever in humans caused by *Salmonella typhi*. In mice, after orally ingested bacteria make their way into circulation, these bacteria are found

disseminated throughout the body, but are particularly prevalent in the liver, spleen and bone marrow (Carter and Collins, 1974). The bacteria traverse both the vasculature and lymphatic system (Carter and Collins, 1974), and are resistant to complement (Joiner, 1988). Wild type *Salmonella* are also resistant to macrophages (Groisman and Saier, 1990) and tend to persist in tissues in which macrophages reside. While these attributes make these bacteria effective as pathogens, they also predispose them for use as delivery vectors with access to a large portion of the human body.

The initial experiments on the biodistribution of *Salmonella* in mice bearing tumors were performed by Pawelek *et al.* (1997). Using the wild type *S. typhimurium* and a purine auxotrophic clone selected to be hyperinvasive *in vitro* (clone YS72), the phenomenon of differential replication within tumors was observed (Table 1). Tumor types that were shown to be targeted by *Salmonella* included melanoma, lung, colon, breast, kidney and liver. Further study by Kops *et al.* (1997) confirm the ability of the bacteria to spread throughout tumors, apparently overcoming the physical barriers of tumors that limit other agents.

Studies of attenuation of virulent *Salmonella* by mutations in metabolic pathways including purines and amino acids suggested that the major difference between the wild type and the YS72 was due to the purine deficiency. Early studies by Bacon *et al.*, (1950; 1951) on purine auxotrophic *Salmonella* indicated that such mutations limit the pathogenesis of these bacteria. Amino acid auxotrophy has also been shown to strongly attenuate *Salmonella* pathogenic capabilities (Hoiseth and Stocker, 1981). In light of this, a reasonable interpretation of the data in Table 1 is that the restricted growth within the liver by the stain YS72 indeed indicates some form of attenuation, and therefore probably due to the purine auxotropy. However, although this strain was attenuated compared to the wild type strain, it was nonetheless too virulent for long term antitumor studies in sensitive mice such as C57B6.

This finding on attenuation was further exploited by making strains of bacteria that were polyauxotrophic (Fig. 1). Selection for polyauxotrophy reduced the toxicity of the strains, increasing the time to death of tumor-bearing mice in a single dose challenge of bacteria (Table 2). Polyauxotrophic strains of *Salmonella* retain high-level replication within tumors and some exhibit tumor to normal tissue rations of greater than 2,000:1 with a total accumulation of greater than 10^9 c.f.u./g (Table 3). This high-level replication within tumors in highly attenuated strains suggests that the acquisition of nutrients abundant in tumors forms a basis for maintaining tumor specificity.

Table 1. Tumor and liver distribution of *Salmonella* at 5 h, and 2 d post i.p. inoculation of 10^6 c.f.u. Points are for C57B6 mice-bearing B16F10 melanoma tumors, representing avg ± S.D. for n = 3 (5 h) and n = 5 (2 d) animals. Modified from Pawelek *et al.* (1997)

	Salmonella/g tissue		
Time Post-Inoc.	Tumor	Liver	Tumor:Liver
5 h			
Wild type	$7.1 \pm 2.2 \times 10^4$	$1.3 \pm 7.6 \times 10^5$	1:2
YS72	$1.2 \pm 1.3 \times 10^5$	$5.5 \pm 4.8 \times 10^4$	2:1
2 d			
Wild type	$6.5 \pm 6.8 \times 10^9$	$2.4 \pm 2.8 \times 10^7$	270:1
YS72	$1.7 \pm 1.2 \times 10^9$	$1.9 \pm 2.3 \times 10^5$	9,000:1

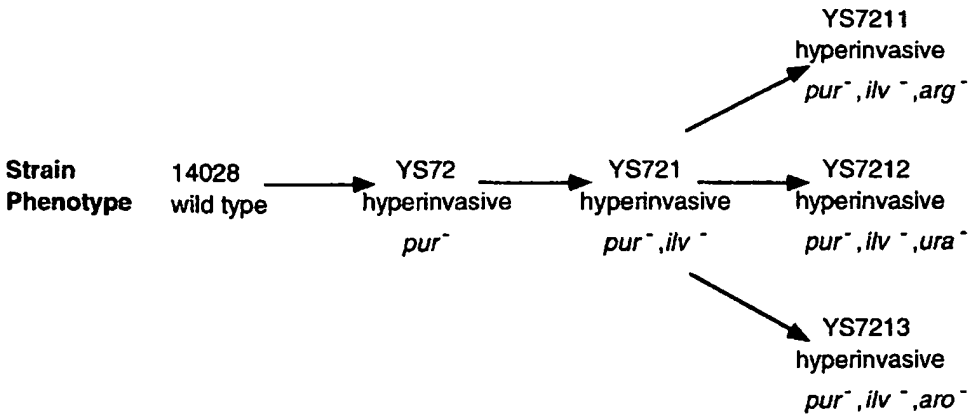

Figure 1. Sequential mutation of the wild type (ATCC 14028) bacteria inducing auxotrophic and polyauxotrophic mutations.

Table 2. Survival of C57B6 mice after introduction of wild type, auxotrophic and polyauxotrophic *Salmonella*. Modified from Pawelek *et al.* (1997)

Strain	Phenotype	Mean Survival (days)
14028	Wild type	3
YS72	*pur*⁻	6
YS721	*pur*⁻, *ilv*⁻	30
YS7211	*pur*⁻, *ilv*⁻, *arg*⁻	>30
YS7212	*pur*⁻, *ilv*⁻, *ura*⁻	>30
YS7213	*pur*⁻, *ilv*⁻, *aro*⁻	>30

Table 3. Tumor and liver distribution of *Salmonella* at 2d and 4d post i.p. inoculation of 10^6c.f.u. Points are for C57B6 mice-bearing B16F10 melanoma tumors, representing avg ± S.D. and n = 5 (2d, 4d) animals. Modified from Pawelek *et al.* (1997)

	Salmonella/g tissue		
Time Post-Inoc.	Tumor	Liver	Tumor:Liver
2d			
YS72	$1.7 \pm 1.2 \times 10^9$	$1.9 \pm 2.3 \times 10^5$	9,000:1
YS721	$8.7 \pm 3.1 \times 10^8$	$4.2 \pm 3.6 \times 10^6$	210:1
YS7211	$3.3 \pm 3.0 \times 10^7$	$8.1 \pm 8.4 \times 10^5$	41:1
YS7212	$3.9 \pm 7.3 \times 10^7$	$1.1 \pm 0.8 \times 10^6$	35:1
YS7213	$1.5 \pm 2.8 \times 10^8$	$4.0 \pm 3.1 \times 10^5$	375:1
4d			
YS72	moribund/dead		
YS721	$3.2 \pm 1.5 \times 10^9$	$4.7 \pm 6.9 \times 10^6$	680:1
YS7211	$1.6 \pm 2.2 \times 10^9$	$6.3 \pm 9.9 \times 10^6$	253:1
YS7212	$1.1 \pm 7.4 \times 10^9$	$5.1 \pm 8.6 \times 10^5$	2,200:1
YS7213	$1.3 \pm 2.5 \times 10^9$	$2.2 \pm 6.9 \times 10^5$	5,900:1

Lipid A modification has now been shown to offer an additional means to attenuate *Salmonella* (Low *et al.*, 1998; Kahn *et al.*, 1998). *Salmonella* deficient in terminal myristolization of lipid A are greatly reduced in their ability to elicit TNFα, the cytokine that initiates septic shock. Yet strains of *Salmonella* bearing this mutation retain both tumor-targeting and antitumor activity. This mutation seems particularly promising in furthering the potential use of systemically administered gram-negative bacterial antitumor agents.

2.1. *Salmonella* Antitumor Activity

These highly attenuated strains of *Salmonella* possess innate antitumor activity (Pawelek *et al.*, 1997). When subcutaneously implanted B16F10 melanoma is staged to day 8 and the bacteria administered systemically by i.p. injection, there is a dramatic slowing of tumor progression (Fig. 2). With one strain YS7212, the treated group survived more than twice as long as the untreated group.

In addition, it has been shown that this same strain inhibits tumor metastasis *in vivo* (Zheng *et al.*, 1997). Since metastatic disease is one of the major causes of death in patients with solid tumors, this aspect of antitumor *Salmonella* is also particularly encouraging.

2.2. *Salmonella* Expression of Prodrug-Converting Enzymes

Bacteria are well developed as protein expression systems. Tumor-targeted *Salmonella* extend the utility of bacteria to include both the expression and the delivery of anticancer therapeutic proteins directly within cancerous tissue. While bacteria do not perform mammalian glycosylation and other protein modifications, there are many effector proteins in which such modifications are unnecessary. The herpes simplex thymidine kinase (HSV TK) is an example of a prodrug converting enzyme that is functionally expressed in bacteria (Garapin *et al.*, 1981). This enzyme activates nucleoside analogues

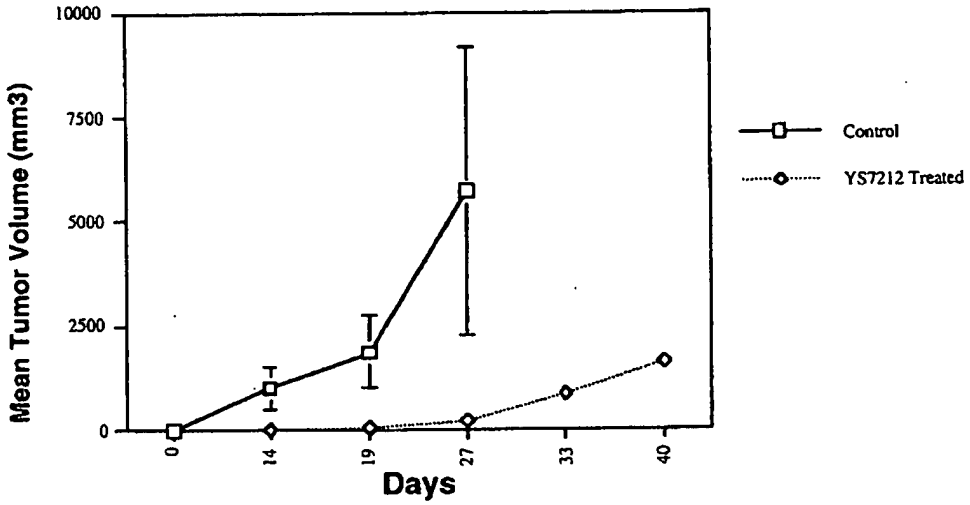

Figure 2. Growth of B16F10 melanoma in C57B6 mice with and without inoculation of strain YS7212. Eight days following introduction of tumor cells, the treated group received 4×10^6 c.f.u. injected i.p. Each point represents the mean ± S.E. for n = 5 animals.

such as acyclovir (ACV) and ganciclovir (GCV). When Pawelek *et al.* (1997) used a strain of *Salmonella* expressing a secreted form of HSV TK in a B16F10 subcutaneous melanoma model, they found 1) that the presence of the plasmid lessened the innate antitumor activity of the bacteria, and 2) that when these bacteria were coadministered with GCV the tumors were 2.5 times smaller than without added GCV. They concluded that these bacteria were capable in delivering a prodrug converting enzyme effective in activating a compound into its chemotherapeutic form.

Another example of prodrug conversion by tumor-targeted *Salmonella* has been studied by King *et al.* (1998). This study demonstrated that *Salmonella* expressing cytosine deaminase effectively convert 5-flurocytosine (5-FC) to 5-fluorouricil (5FU) within tumors and resulted in antitumor activity against subcutaneously implanted B16F10 melanoma.

Taken together, these studies indicate that *Salmonella* may be useful in expressing a variety of prodrug converting enzymes directly within tumors resulting in antitumor effects when given together with their appropriate prodrug, and therefore applicable in treatment of a wide range of tumor types.

3. SUMMARY

Genetically engineered *Salmonella* offer an intriguing new approach to selectively target solid tumors, including melanoma, lung, colon, breast, kidney and liver. These bacteria target tumors after systemic administration and selectively replicate within them. Specificity for tumors is often more than 1,000 times greater than for any other tissue. Auxotrophic mutations make these bacteria highly safe and form the basis for maintaining tumor specificity. An altered lipid greatly reduces the potential for septic shock yet also retains the antitumor properties of these bacteria. These bacteria have innate antitumor activity towards both primary and metastatic tumors and the ability to deliver proteins capable of activating chemotherapeutic agents directly within tumors. The delay in tumor growth results in mice that survive up to twice as long. These bacteria are susceptible to a wide range of antibiotics, allowing external control of the vector after administration. The combination of these features within a single vector seems especially surprising considering their unlikely source.

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EXHIBIT B

Gene Therapy (1997) 4, 791–796

Anaerobic bacteria as a gene delivery system that is controlled by the tumor microenvironment

MJ Lemmon[1], P van Zijl[1], ME Fox[1], ML Mauchline[2], AJ Giaccia[1], NP Minton[2] and JM Brown[1]
[1]*Mayer Cancer Biology Research Laboratory, Department of Radiation Oncology, Stanford University School of Medicine, Stanford CA 94305, USA; and* [2]*Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire, UK*

A fundamental obstacle in gene therapy for cancer treatment is the specific delivery of an anticancer gene product to a solid tumor. Although several strategies exist to control gene expression once a vector is directly introduced into a tumor, as yet no systemic delivery system exists that specifically targets solid tumors. Nonpathogenic, obligate anaerobic bacteria of the genus Clostridium have been used experimentally as anticancer agents because of their selective growth in the hypoxic regions of solid tumors after systemic application. In this report we further describe a novel approach to cancer gene therapy in which genetically engineered clostridia are used as tumor-specific vectors for the delivery of antitumor genes. We have introduced into a strain of C. beijerinckii the gene for an E. coli nitroreductase known to activate the nontoxic prodrug CB 1954 to a toxic anticancer drug. Nitroreductase produced by these clostridia enhanced the killing of tumor cells in vitro by CB 1954, by a factor of 22. To demonstrate the specificity of this approach for tumor targeting, we intravenously injected the inactive spore form of C. beijerinckii, which upon transition to a reproductive state will express the E. coli nitroreductase gene. Nitroreductase activity was detectable in 10 of 10 tumors during the first 5 days after intravenous injection of inactive clostridial spores, indicating a rapid transition from spore to reproductive state. Tumors harboring clostridial spores which did not possess the E. coli nitroreductase gene were devoid of nitroreductase activity. Most importantly, E. coli nitroreductase protein was not found in a large survey of normal mouse tissues following intravenous injection of nitroreductase containing clostridia, strongly suggesting that obligate anaerobic bacteria such as clostridia can be utilized as highly specific gene delivery vectors for cancer therapy.

Keywords: *gene therapy; nitroreductase; clostridia; tumor hypoxia; enzyme prodrug therapy*

Introduction

It has been known for several decades that certain species of anaerobic bacteria, of the genus *Clostridium*, can selectively germinate and grow in the hypoxic regions of solid tumors after intravenous injection. These hypoxic regions are characteristic of solid tumors in rodents[1] and occur with high frequency in many human tumors.[2]

The tumor selective germination of clostridia was dramatically demonstrated by Malmgren and Flanigen[3] with *C. tetani*, the causative agent of tetanus. Mice, when injected intravenously with spores of *C. tetani*, remained healthy unless they had tumors, in which case death by tetanus resulted within 48 h. This occurred because the bacteria were able to germinate in the tumors, thereby releasing toxins systemically. Möse and colleagues[4] later isolated a nonpathogenic strain of *C. sporogenes* called M55 (this strain was later renamed *C. oncolyticum*). These and subsequent authors were interested in these nonpathogenic bacteria as anticancer agents because their germination in tumors resulted in a partial tumor lysis.[5,6] More importantly, it was demonstrated that the clostridia germinated solely in the tumor after intravenous injection of the inactive spore form.[7]

Correspondence: JM Brown
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Clinical trials were initiated using *C. oncolyticum* as an antitumor agent. Cancer patients, particularly those with glioblastoma multiforme, were injected intravenously with up to 10^{10} *C. oncolyticum* spores.[8,9] As with the animal tumors, most of the human malignancies experienced partial lysis with no evidence of clostridial germination or tissue destruction in the surrounding normal tissue. Furthermore, with the exception of mild to moderate fever, the patients suffered no ill effects from the injection of these organisms in large numbers. Unfortunately, *C. oncolyticum* by itself did not produce significant antitumor activity, so the trials were discontinued.

We hypothesized that clostridia could be genetically manipulated, thereby exploiting tumor hypoxia for a new form of gene therapy (Figure 1). We have previously reported on the genetic manipulation of *C. beijerinckii* to produce the *E. coli* enzymes cytosine deaminase[10] and nitroreductase,[11] enzymes known to activate nontoxic prodrugs to toxic anticancer drugs. This article describes the selective production of *E. coli* nitroreductase in murine tumors *in situ* following the intravenous injection of genetically modified *C. beijerinckii* spores.

E. coli nitroreductase activates the prodrug CB 1954 (5-aziridino-2,4-dinitrobenzamide) to a bifunctional alkylating agent known to cause DNA interstrand cross-links (reviewed in Ref. 12). This 24 kDa enzyme, similar in sequence to the 'classic nitroreductase' of *Salmonella typhimurium*, was first isolated and sequenced by

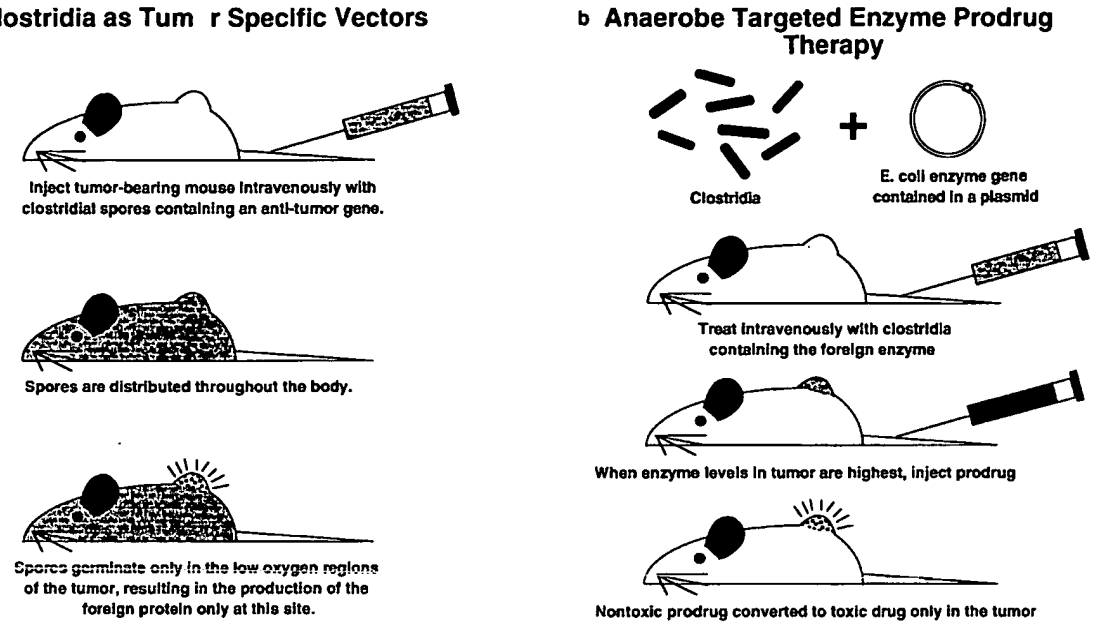

Figure 1 (a) Rationale for using clostridial spores as tumor-specific vectors. (b) Scheme for anaerobe targeted enzyme prodrug therapy.

Anlezark and colleagues[13] from *E. coli* B. CB 1954, a monofunctional alkylating agent originally synthesized over 25 years ago,[14] has potent activity against Walker 256 rat carcinoma cells due to efficient drug reduction to the toxic bifunctional alkylating agent by rat (but not human) DT-diaphorase.[15] *E. coli* B nitroreductase is approximately 30-fold more efficient than rat DT-diaphorase at producing the active form of CB 1954.[16]

As described above, most of the early clostridia tumor studies were performed with *C. oncolyticum* M55, a strain of *C. sporogenes*. However, selective germination in tumors after intravenous injection has also been reported with other species, including *C. acetobutylicum*,[17] which for many decades has been used as a commercial producer of acetone and butanol. Since plasmid vectors and transformation procedures are available for *C. acetobutylicum* and not for *C. sporogenes*, we used a strain of *C. acetobutylicum* (recently reclassified as *C. beijerinckii*) in our studies. In this article, we present evidence for the tumor-specific expression of *E. coli* B nitroreductase, using *C. beijerinckii* as the gene delivery vector.

Results

Selective growth of unmodified clostridia in tumors

To demonstrate that systemically applied clostridia grow selectively in tumors, EMT6 tumor-bearing mice were injected intravenously with 10^8 wild-type clostridial spores, killed 24 h later, and examined for the presence of Gram-positive clostridial rods in both tumors and normal tissues. Tumors exhibited a heavy infestation of Gram-positive staining rods in and surrounding the region of central necrosis, a region presumed to be at low oxygen tension (Figure 2b, c).[18] A light scattering of Gram-positive rods was infrequently found elsewhere in the tumors. Control tumors from mice injected with phosphate-buffered saline alone stained negative for the presence of Gram-positive rods (Figure 2a). In addition, Gram staining of histological sections indicated that clostridia did not germinate in normal tissues, including heart, kidney, liver, lung and spleen (Figure 2d).

Tumor cell killing by clostridia-activated CB 1954 in vitro

Survival of EMT6 tumor cells in culture exposed to the prodrug CB 1954 was examined by exposing tumor cells to media in which clostridia, with or without the nitroreductase gene, had been grown. Supernatant from clostridia containing the nitroreductase plasmid (pNTR500F) expressed 100–200 mU nitroreductase activity per ml, as assayed by following the reduction of dichlorophenol-indophenol with a spectrophotometer. There was a 22-fold increase in cell killing by CB 1954 when the clostridia-produced nitroreductase was present (Figure 3a). Similar cell survival results were obtained with SCCVII mouse tumor cells (data not shown). Reduction of CB 1954 by the nitroreductase-producing clostridia but not by control clostridia was confirmed by measuring loss of the parent drug with high pressure liquid chromatography in samples taken from these survival experiments (Figure 3b).

Immunoblot detection of nitroreductase in tumors

Mice bearing EMT6 tumors were injected intravenously with spores containing the nitroreductase plasmid, or with spores containing the empty plasmid (pMTL500F). Nitroreductase protein was detected by immunoblot analysis in nine of 10 tumors removed 1–5 days after intravenous injection of clostridia containing the nitroreductase gene (Figure 4a). The amount of nitroreductase correlated with the numbers of viable clostridia found in these tumors, which ranged from 10^5–10^6 per gram of tumor, and on average 78% of the bacteria contained the plasmid (±27, 1 s.d.m.). These bacteria were almost all (<96%) in the non-spore form, indicating germination of the spores in the tumors as early as 24 h after spore injection. Plasmid control tumors, which had similar numbers

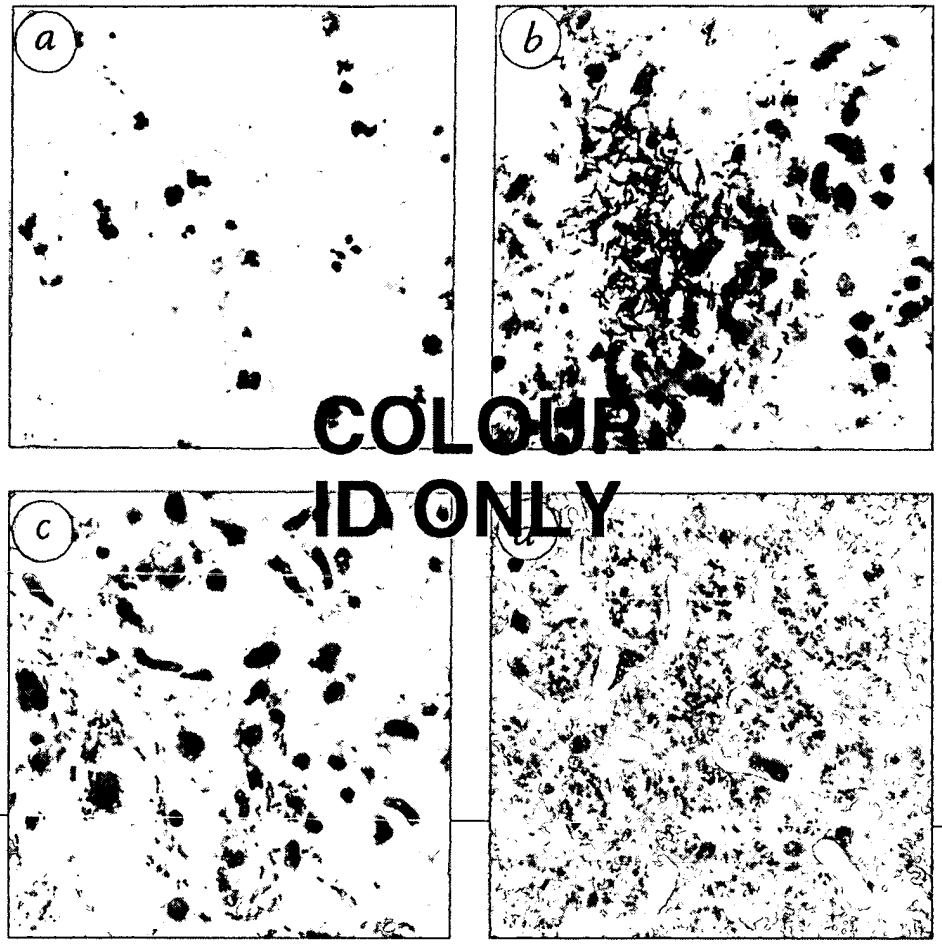

Figure 2 Photomicrograph of EMT6 tumor and normal mouse tissue sections stained by the Gram-Twort method. (a) Necrotic tumor region from a mouse injected with saline alone that was stained for Gram-positive rods 24 h after injection. (b) Small necrotic tumor region from a mouse injected with C. beijerinckii spores stained for Gram-positive rods 24 h after injection. (c) Border between necrotic and non-necrotic regions of a tumor from a mouse injected with C. beijerinckii spores stained for Gram-positive rods 24 h after injection. (d) Liver section from a mouse injected with C. beijerinckii spores stained for Gram-positive rods 24 h after injection, with no evidence of bacteria. All photomicrographs original ×600 magnification.

of viable bacteria at the time of harvest, had no detectable nitroreductase protein by immunoblot assay (Figure 4a).

Significant nitroreductase activity was detected in protein preparations of all 10 tumors obtained from mice injected with clostridia containing the nitroreductase plasmid (Figure 4a). Nitroreductase activity in plasmid control tumors was at least several orders of magnitude lower and could not be detected (Figure 4a). The enzyme activity in these tumors *in vivo* was roughly equivalent on an activity per volume basis to that seen in the medium of stationary phase clostridia growing in an anaerobic chamber (ie the activity in a 500 mm^3 tumor was approximately the same as the activity in 0.5 ml of supernatant from a nitroreductase-producing clostridial culture).

Immunoblot detection of nitroreductase in normal tissues

Normal tissues from the five mice with the strongest nitroreductase protein levels in their tumors were examined by immunoblot assay. Tissues examined were brain, heart, upper intestine (jejunum region), lower intestine with resident feces, kidney, liver, lung, skeletal muscle (gastrocnemius) and spleen. Of these tissues, immunoblots for all except hearts are shown in Figure 4b. No detectable nitroreductase protein was present in any of these tissues. A protein with weak cross-reactivity that possesses a similar molecular weight to nitroreductase was detected in the small intestine and skeletal muscle. However, these weak cross-reactivities were also found in untreated mice (data not shown). Protein extracts from heart tissue exhibited antibody cross-reactivity similar in intensity and molecular weight to that found in tumors (Figure 4c). Since cross-reactivity was also present in immunoblots from protein preparations of hearts from mice that had been injected with plasmid control clostridia, as well as from untreated mice (Figure 4c), we conclude that this protein in mouse heart is naturally occurring and is not *E. coli* nitroreductase. Thus, production of nitroreductase by clostridia after intravenous injection into tumor-bearing mice was found to be specific to the tumors.

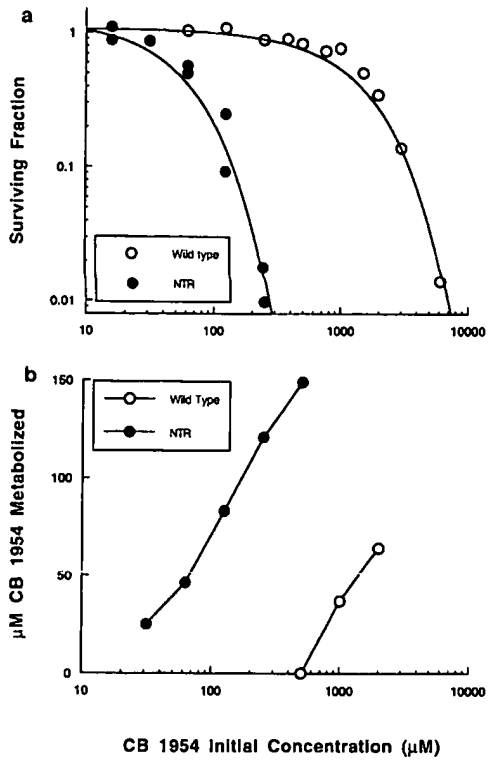

Figure 3 EMT6 tumor cell survival and drug metabolism in the presence of the prodrug CB 1954 and clostridia-produced nitroreductase. (a) EMT6 survival after a 2 h exposure to CB 1954 in combination with clostridial supernatant from wild-type or nitroreductase (NTR) gene containing clostridia. Each data point represents one independent survival measurement. Two experiments are combined. (b) CB 1954 metabolism after a 2 h exposure as described in (a) as measured by high pressure liquid chromatography (HPLC). The μM CB 1954 metabolized was calculated as: CB 1954 dose – CB 1954 remaining. Samples for HPLC analysis are from the same experiments as in (a).

Discussion

The central problem for gene therapy of cancer is the lack of specificity of current delivery systems. To date no technique exists for the specific delivery of novel genes to tumors when the delivery system is systemically injected. We further describe a novel approach to this problem, the production of antitumor agents by genetically modified obligate anaerobic bacteria. We have demonstrated a highly tumor-specific localization of the product of clostridial gene expression in tumor-bearing mice after systemic application. The lack of clostridial gene products in normal tissues indicates not only that the oxygen tension in these tissues is sufficiently high to prevent clostridial germination, but also that the gene products do not circulate from the tumor to other tissues via the bloodstream or other routes. The only requirement for success of this gene therapy technique in the clinic should be the presence of hypoxia in the tumors being treated. Disseminated as well as primary disease should be amenable to treatment as long as regions of hypoxia are present – in fact growth of clostridia in metastatic lesions of human cancer patients after intravenous injection of clostridial spores has already been demonstrated.[8]

This gene delivery system is not only tumor specific, it is also nontoxic. The lack of pathogenicity of these organisms in humans was initially demonstrated by Möse *et al* (cited in Ref. 8) when the authors injected themselves with *C. oncolyticum* spores, and experienced a mild fever as the only side-effect. The lack of pathogenicity was also substantiated in clinical trials with *C. oncolyticum*[8,9] and also in more recent trials with a strain of *C. beijerinckii*.[19]

As a further safeguard for this gene delivery system, we chose to express in clostridia a drug-activating enzyme rather than a protein that could kill tumor cells directly, and which might produce host toxicity if it found its way into the systemic circulation. A further advantage of expressing drug-activating enzymes is its amplification potential, since one enzyme molecule can activate many molecules of prodrug. The nitroreductase/CB 1954 enzyme prodrug system is one of several enzyme prodrug schemes that are currently being investigated in other laboratories (reviewed in Ref. 20).

In summary, we have demonstrated the principle that a nonpathogenic species of clostridia can be engineered to express a foreign gene product selectively in a solid tumor after intravenous injection of the inactive spore form. When these genetically engineered clostridia are introduced systemically into tumor-bearing mice, the product of the introduced enzyme gene is found only in the tumors, presumably due to the hypoxic environment required for growth of these bacteria. We believe that this new delivery system holds promise for gene therapy of solid tumors, because of its lack of toxicity and highly selective growth in tumors.

Materials and methods

Tumor cell survival assays

The prodrug CB 1954 was obtained from Dr Richard Knox and Dr D Wilman, The Institute of Cancer Research, Sutton, UK. EMT6 tumor cells were seeded into 60 mm plastic tissue culture dishes, allowed to grow for 2 days, and treated when about 30–50% confluent by replacing the media with supernatant from *C. beijerinckii* cultures. Following overnight growth, *C. beijerinckii* containing the plasmid with or without the nitroreductase gene were diluted 1:10 in bacterial media and allowed to grow to stationary phase, as determined by optical density at 600 nm. This is the stage of bacterial growth where we found the largest quantity of active nitroreductase in the bacterial media (data not shown). The clostridial cultures were then centrifuged and the pH of the supernatant, which was typically about 5.0, was adjusted to 7.4 with NaOH. The tumor cells were treated for 2 h at 37°C with clostridial supernatant in combination with CB 1954 and 500 μM NADH. After treatment, the bacterial supernatant was removed, the tumor cells were washed three times with PBS, trypsinized, counted with a Coulter counter (Hialeah, FL, USA) and plated at various dilutions in fresh medium to assay for clonogenic survival. After an 8 day incubation at 37°C, tumor cells were stained with crystal violet, and colonies containing at least 50 cells were counted. Clonogenic cell survival was calculated relative to that of untreated controls. Samples of supernatant were analyzed at the end of treatment for metabolism of CB 1954 by high pressure liquid chromatography (HPLC) by a procedure similar to that of Sunters *et al*,[21] using a Water's Maxima system (Milford, MA,

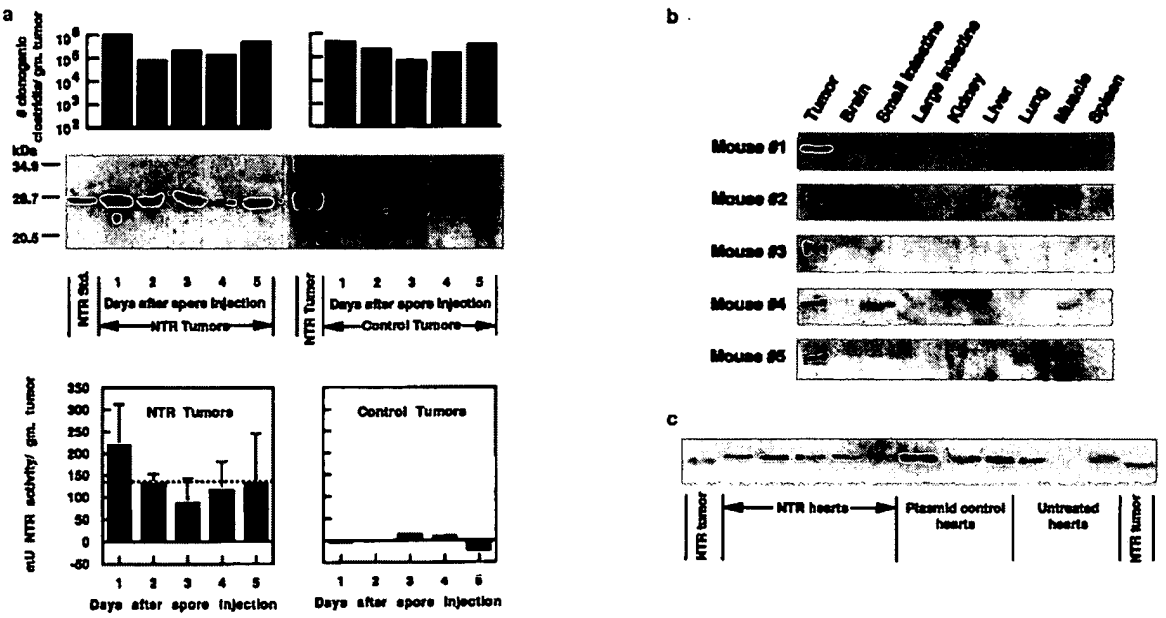

Figure 4 (a) Comparison of the number of viable vegetative clostridia with nitroreductase protein and activity levels in tumors removed from mice on days 1–5 after injection of clostridial spores containing the nitroreductase gene or empty plasmid vector. Purified E. coli nitroreductase standard (NTR std.–4 ng) is included for comparison. 50 μg of protein extract from individual tumors was electrophoresed, immunoblotted and probed with E. coli B nitroreductase antibody. Nitroreductase activity is shown in 10 tumors removed from mice 1–5 days after injection with clostridial spores containing the nitroreductase gene, or from five tumors after injection with clostridial spores containing only the empty plasmid vector. Mean nitroreductase activity is represented by a dotted line and error bars represent one standard error of the mean. (b) Expression of E. coli B nitroreductase protein in normal tissues excised from mice 1–5 days after injection with genetically engineered clostridial spores. 25 μg of protein extract from each tissue was analyzed by immunoblotting with the same antibody used in analysis of tumor extracts in (a). (c) Immunoblots of protein extracts from heart tissue of mice 1–5 days after injection with spores of clostridia containing the nitroreductase gene, with spores of clostridia containing empty plasmid vector, or sterile PBS. 50 μg of protein extract was analyzed from 11 individual hearts. Nitroreductase protein in extracts of tumor tissue is included for comparison.

USA). Samples were diluted in mobile phase buffer (25% methanol, 0.1 M amonium acetate, pH 7.0), and separated on a C_{18} column at a flow rate of 5 ml/min. The absorbance of the effluent was monitored at 325 nm.

Spore induction and systemic delivery to tumor-bearing mice

C. beijerinckii NCIMB 8052 was grown in an anaerobic chamber (Sheldon Manufacturing Bactron II, Cornelius, OR, USA) at 37°C in 2 × YTG media (16 g tryptone, 10 g peptone, 5 g NaCl and 5 g glucose per liter). Sporulation was induced by either the method of Kashket and Cao,[22] or the bacteria were left to sporulate for 2–3 weeks on 1.5% agar plates of 2 × YTG media. Following spore harvest, contaminating bacterial rods were removed according to the protocol of Kihm *et al*,[23] after which the spore suspensions were washed and resuspended in phosphate-buffered saline for mouse injection. Balb/c female mice, 2.5–3 months old, were injected intradermally in the sacral region of the back with 2×10^5 EMT6 mouse mammary carcinoma cells in 50 μl of Waymouth's medium. The mice were bred and raised under specific pathogen-free conditions in the Stanford Medical School Research Animal Facility, and were provided sterilized water and rodent chow *ad libitum*. Bacterial spores (10^8) were injected intravenously when the tumors were approximately 200 mm^3. For histological analysis, the animals were killed 24 h after spore injection, tumors and normal tissues were excised, sectioned in paraffin, and stained with a modified Gram stain.[24]

Introduction and selection for the nitroreductase gene

E. coli B nitroreductase was isolated by polymerase chain reaction and cloned into the *NdeI* and *PstI* restriction sites of the multiple cloning region of the pMTL500F shuttle vector, resulting in constitutive expression of the nitroreductase gene in clostridia.[11] This plasmid is called pNTR500F. 2 × YTG medium was supplemented with 20 μg/ml erythromycin or clarythromycin for selection of bacteria containing the introduced plasmids, which have an erythromycin resistance gene. Clostridial spores, containing either the control or nitroreductase plasmid, were injected intravenously into tumor-bearing mice as described above. Mice were also given subcutaneous injections twice daily of 50 mg/kg azithromycin (Pfizer, Groton, CT, USA) to maintain selective pressure for plasmid retention.[25] One to five days after intravenous injection of spores, mice were killed by cervical dislocation and their tumors and normal tissues were removed. Tumor samples were plated under anaerobic conditions before and after a 70°C 10 min heat shock to determine the number of viable bacteria and spores in each specimen, respectively.

Analysis of E. coli B nitroreductase protein and activity in tumor and normal tissue

Fifty micrograms of protein extracts and 5 ng of purified nitroreductase protein[26] were electrophoresed on a 12.5% SDS-PAGE polyacrylamide gel, transferred to nitrocellulose (Hybond ECL; Amersham, Arlington Heights, IL, USA), and stained with Coomassie brilliant blue to

ensure uniform transfer of proteins. Nitrocellulose membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS 10 mM Tris, 150 mM NaCl, pH 8.0) overnight at 4°C, and then incubated at room temperature for 3 h with a 1:5000 dilution of a polyclonal antibody to *E. coli* B nitroreductase (supplied by Dr Richard Knox, The Institute of Cancer Research). Membranes were washed in TBS and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG, and autoradiographed with a chemoluminescence detection system according to the manufacturer's instructions (ECL detection system, Amersham). Nitroreductase activity of samples was measured spectrophotometrically by assaying for the reduction of dichlorophenol-indophenol (DCPIP).[27] All samples were run in duplicate, both with and without dicumarol (3,3'-methylene-bis(4-hydroxycoumarin)), which inhibits the activity of nitroreductase. Nitroreductase activity was expressed as the difference between the activity without dicumarol and the activity with dicumarol. Purified nitroreductase was used as a positive control in activity assays.

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EXHIBIT C

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Delivery systems intended for in vivo gene therapy of cancer: targeting and replication competent viral vectors

Evanthia Galanis [a,b,*], Richard Vile [b], Stephen J. Russell [b]

[a] *Department of Oncology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA*
[b] *Molecular Medicine Program, Mayo clinic, 200 First Street SW, Rochester, MN 55905, USA*

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Contents

* Corresponding author. Tel.: +1-507-2843731; fax: +1-507-5380823.
E-mail address: galanis.evanthia@mayo.edu (E. Galanis).

Abstract

Cancer gene therapy represents one of the most rapidly evolving areas in pre-clinical and clinical cancer research. Application of gene transfer techniques in clinical trials has made increasingly obvious that several issues will need to be addressed prior to meaningful incorporation of gene therapy in the care of cancer patients. Two of the most important problems to overcome are lack of selectivity of the existing vectors and low efficiency of gene transfer. This review focuses on use of targeting and replication competent vectors in order to overcome these obstacles. Targeted gene therapy of malignancies can be achieved through vector targeting or transcriptional targeting and can improve the therapeutic index of gene transfer by preventing damage of normal tissues, an important requirement if systemic gene delivery is contemplated. Replication competent viral vectors can improve the efficiency of gene transfer. Provisionally replicating viruses can also improve the therapeutic index by targeting toxicity to tumor cells. A variety of provisionally replicating viruses, such as the attenuated adenovirus ONYX-015, the adenovirus CN706 that selectively replicates in prostate cancer cells, the double mutant herpes simplex virus G207, the human reovirus, and the Newcastle disease virus are currently in clinical trials. Early clinical results and limitations in the application of these vectors are discussed.

Keywords: Cancer gene therapy; Targeting; Replicating vectors

1. Introduction

The development of recombinant DNA technologies has introduced a new insight into the molecular basis of neoplasia [1]. Detection of common alterations shared by multiple cancers has increased optimism that molecular genetics may lead to improved clinical cancer care. The generation of better gene transfer systems capable of modifying human tissues has strengthened the idea that gene therapy can be used for the treatment of cancer as well [2–4] and has led to a large number of pre-clinical investigations and clinical trials that explore the potential of gene transfer in cancer therapy [5]. With more than 200 cancer gene therapy trials approved worldwide since the early 1990s it has become increasingly apparent that the existing gene delivery vehicles (vectors) will need significant improvement prior to any meaningful incorporation of gene transfer techniques into the care of cancer patients. Two of the most important obstacles to overcome are lack of selectivity of the existing vectors and low efficiency of gene transfer. Overcoming these two obstacles would represent a major step toward the realization of targetable vectors that can be intravenously administered – an important goal given the systemic nature of most malignancies.

This review will focus on strategies to improve efficacy and selectivity of gene transfer approaches in cancer gene therapy by employing targeting and replication competent vectors.

2. Targeting

Targeted gene therapy of malignancies can be achieved through (1) vector targeting, and (2) targeted gene expression. Targeting can improve the therapeutic index of gene transfer by preventing damage of healthy tissues and decreasing the risk of germ line transduction. Although less important with intratumoral gene delivery, it becomes crucial when systemic gene transfer is contemplated.

2.1. Vector targeting

Vector targeting efforts involve both viral and non-viral vectors (i.e., liposomes, molecular conjugates). In this review, we will limit the discussion to targeting of viral vectors, including retrovirus, adenovirus, adeno-associated virus (AAV), Sindbis virus, and Herpes simplex virus type 1 (HSV-1).

2.1.1. Retroviral targeting

Retroviruses are enveloped viruses that carry a core containing RNA as their genetic information. The infection process is initiated by an interaction between the viral envelope (surface) protein and one or more specific receptor molecules located in the plasma membrane of the cell to be infected. Most of the specificity of the retroviruses is primarily dictated by the nature of envelope glycoproteins that they carry [6]; each type of retrovirus recognizes a different receptor, but only some of these receptors have been cloned and characterized [7,8]. For example, the two main species of murine leukemia virus (MLV) based retroviral vectors, ecotropic (infect murine cells only), and amphotropic (infect murine and human cells) differ in specificity as a result of differences between the two env proteins. In the case of ecotropic viruses, that allows binding of viral particles to a cationic amino acid receptor (Ram-1 or CAT), while in the case of amphotropic retroviruses,

the receptor is a phosphate transporter (Ran-1 or GLVR-2). Not all cell types of a given species are infected equally well with retroviruses: many cell types including certain cells of therapeutic interest such as hematopoietic progenitors are poorly sensitive to retroviral transduction. Several strategies can be used to modify the tropism of retroviruses. Examples include replacing the envelope glycoprotein of one retrovirus with that of another retrovirus, an approach called pseudotyping, genetic modification of the retroviral envelope glycoprotein, or coupling of bifunctional bridging agents to the viral surface in order to modify the specificity of infection (Fig. 1).

2.1.1.1. Pseudotyping. Although in most cases pseudotyping broadens rather than restricts the selectivity of viral infection, it is included here since it can be used as a means of allowing infection of refractory cells.

Because *cis*-acting functions and *trans*-acting functions (such as the gag, pol, and env proteins) can be separated in retroviral packaging cell lines, pseudotyping can be accomplished through replacement of the usual viral env expression vector by another one.

MLV-based virions can be pseudotyped with the G glycoprotein of the vesicular stomatitis virus (VSV). This provides a promising approach to overcoming limitations such as refractory target cells or low production titers of retroviruses because VSV-G-pseudotyped vectors recognize common membrane phospholipids and infect a wide spectrum of animal cells. In addition, concentration to titers as high as 10^9 infectious particles/ml can be achieved by ultracentrifugation [9].

MLV-based virions require cell division for integration into the infected cell genome. This prohibits viral integration into non-dividing cells. Of note, quiescent cells represent a significant percentage of even the most aggressive tumors. In contrast, lentiviruses are relatively independent of cell division and, therefore, can infect quiescent cells. Nevertheless, the restricted binding specificity of the HIV envelope glycoprotein allows efficient infection of only certain cell types such as T-cells and macrophages and limits the lentiviral applicability as gene transfer vehicles. Pseudotyping can increase the potential therapeutic utility of lentiviral vectors by expanding their tropism. For example, pseudotyping of HIV-based vectors with the amphotropic MLV env allowed gene transfer into human cells, with significant therapeutic potential such as skin fibroblasts and peripheral blood CD-34 positive cells [10]. In addition, pseudotyping of HIV-1 with the VSV envelope glycoprotein G (VSV-G) enabled the virus to infect a wide

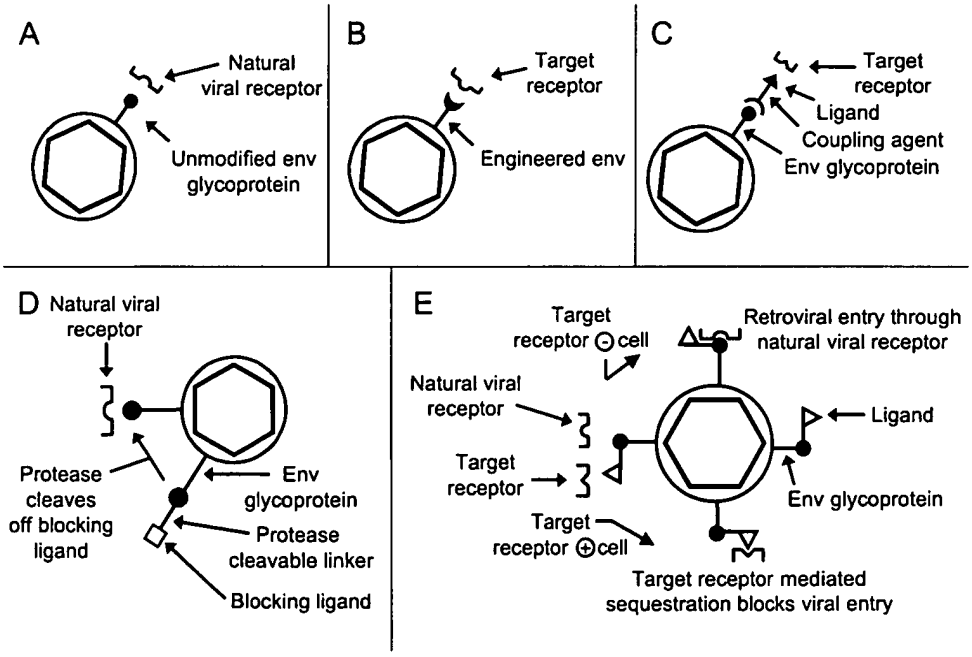

Fig. 1. Retroviral targeting strategies. (A) Retroviral entry through the natural viral receptor. (B) Engineered envelope glycoprotein mediates entry through target receptor. (C) Bifunctional crosslinkers that recognize both the viral envelope and target receptors on the cell surface can retarget retroviral entry. (D) Protease mediated targeting; the infectivity of the (amphotropic) retroviral vector is blocked by a blocking ligand, but it can be restored when proteases present in the environment cleave off the blocking ligand through a protease cleavable linker. (E) Inverse targeting: a polypeptide ligand is displayed at the retroviral envelope that can lead to receptor-mediated sequestration. Retroviral entry is blocked in cells that express the target receptor since interaction of the displayed polypeptide with the target receptor leads into viral sequestration through a degradative pathway. In contrast, in cells that do not express the target receptor, viral entry occurs through the natural receptor.

range of cell types and allowed viral concentration to much higher titers [11].

2.1.1.2. Genetic modification of the retroviral envelope glycoprotein. Retroviral envelope glycoproteins are synthesized as precursors that are then proteolytically processed to mature surface and transmembrane proteins (TM) [7], which form heterodimers. Viral infection is initiated by surface binding to the viral cellular receptor via peptide motifs located in the surface N-terminal moiety. TM anchors the heterodimer to the viral envelope. In addition, after surface binding to the viral cell surface receptor, a conformational change occurs and the fusion peptide at the C-terminus of the TM is exposed. That leads to fusion between viral and cellular membranes and viral infection. Different genetic modifications have been described for modifying the binding specificity of different retroviral env glycoproteins.

Engineering of the ecotropic murine leukemia virus env protein. Different polypeptides have been displayed at the N-terminal env of the ecotropic Moloney MLV surface in an attempt to extend the host range of ecotropic MLV. Examples include erythropoietin [12], heregulin [13], and CD4 [14]. This allowed infection of human cells via the erythropoietin receptor, two members of the epidermal growth factor receptor (EGFR) family (using heregulin) and human cells expressing HIV-1 (using CD4), although at such low efficiency that the utility of the approach is highly questionable. Of note, co-expression of the wild-type env protein was necessary for infection to occur, possibly because incorporation of the engineered env protein in the virions can be facilitated by the oligomerization of both wild-type and chimeric env proteins.

Another approach for engineering of the ecotropic MLV env protein involves the display of different polypeptide binding domains to the N-terminus of the ecotropic Moloney MLV surface protein. Examples include the N-terminal moiety of the amphotropic MLV env [15,16], single chain antibodies (ScAb) recognizing different cell surface receptors [17–20], heregulin [21], and epidermal growth factor (EGF) [15]. In only a minority of those attempts, the specificity of infection was redefined [22,23], however, with infection efficiencies dramatically lower than those obtained with viruses having wild-type amphotropic envelopes.

Engineering of the amphotropic murine leukemia virus env protein. Limited experiments to target human cells have been conducted using ecotropic MLVs or avian viruses because their receptors do not exist naturally in human cells. More recently, engineering of the 4070A amphotropic MLV envelope glycoprotein has been explored. Interestingly, when amphotropic MLV env proteins are engineered to express EGF-4070A envelope fusion proteins, they can infect human epidermal growth factor receptor (EGFR) negative but not EGFR positive cells [15], a strategy known as inverse targeting. In EGFR negative cells, infection occurs through the amphotropic receptor. In EGFR positive cells, however, the viral particles bind preferentially to the EGFR rather than the amphotropic receptor. The latter sequesters them through a degradative pathway, thus preventing infection. A two-step targeted infection strategy has been built on this principle. EGF was displayed at the N-terminus of the amphotropic envelope and connected with the envelope glycoprotein via a short peptide linker containing the cleavage site for factor Xa-protease [23]. EGFR positive cells were exposed to the virions and subsequently treated with factor Xa. As a result, the EGF was cleaved, and infection was achieved through the natural amphotropic receptors. This approach could be applicable in vivo in order to achieve increased concentration of retroviruses at target cells. Then, appropriate proteases could be administered systematically in order to activate retroviral particles localized on the target cells. Alternatively, this system could make use of proteases normally circulating in plasma or found in high concentration in the tumor microenvironment such as matrix metalloproteinases. Peng et al. [24] displayed blocking domains on retroviral vector particles as an MMP cleavable N-terminal extensions of the 4070A MLV envelope glycoprotein. In the presence of exogenous MMPs, cleavage activation occurred that was partially blocked by MMP inhibitors. In vivo, the MMP-targeted vectors showed strong selectivity for MMP-rich tumor xenografts [25].

Engineering of the avian leukosis virus env protein. Instead of gross modifications such as substitution of whole env domains or insertions of large peptide motifs, more subtle modifications of receptor binding domains have also been made in various retroviral envelope proteins. The aim was to limit alterations in the folding of env proteins and, thereby, to preserve important functions such as processing, incorporation into viral particles or fusion activity. A small peptide containing a RGD motif which binds specifically to integrins was inserted at the binding domain of the avian leukosis virus (ALV) env protein at the position predicted by computer analysis to tolerate a polypeptide insertion [22]. This insertion led to low efficiency infection of human cells.

Engineering of the spleen necrosis virus env. The spleen necrosis virus (SNV) envelope was engineered to display ScAb against (a) Her2/*neu*, a member of the EGFR family of receptors that is overexpressed in 20–30% of breast and ovarian cancers but also displayed on numerous other cell types; (b) CD34, expressed in human stem cells and other progenitor cells of the hematopoietic system; (c) a carcinoembryonic

antigen cross-reacting cell surface protein; and (d) transferrin receptor (expressed on the surface of proliferating cells). Titers above 10^5 CFU/ml were obtained. Cell type specific infectivity was achieved that in interference assays were ScAb and epitope specific [26]. Non-human packaging cell lines were employed for the production of the engineered SNV particles. Since vectors produced from non-human packaging cells tend to be inactivated by human complement, development of human packaging cells for SNV production along with the use of ScAb of even higher specificity would facilitate the clinical translation of the SNV-derived targeting system.

2.1.1.3. Coupling agents to the viral surface to modify the specificity of infection. Use of bifunctional bridging agents recognizing both the retrovirus and the chosen cell surface molecule [27–29] provided evidence that retroviruses can enter cells via cell surface molecules that are not viral receptors. Such bridging agents were used to infect human cells that were naturally resistant to ecotropic MLV-based vectors. The agents used usually consisted of an antibody to the MLV envelope protein connected to another antibody or a growth factor that would bind to the appropriate receptor such as the EGFR, the insulin receptor, or major histocompatibility complex (MHC) class I and class II molecules. Unfortunately, infection efficiencies were extremely low [28,29], thus emphasizing that efficient binding of a retrovirus to a membrane molecule other than the natural receptor, cannot guarantee successful cell infection. In an alternative approach, the extracellular domain of TVA which is the cellular receptor for subgroup A avian leukosis viruses (ALV-A) was linked to the mature form of human EGF. A TVA–EGF fusion protein pre-bound to mouse fibroblasts expressing human EGF rendered these cells highly susceptible to infection by ALV-A vectors in an EGF specific manner [30]. Although this appears to be an interesting concept for targeting retroviral infection to specific cell types, its applicability in human disease is limited by the fact that mammalian host cell non-permissiveness cannot always be reversed simply by making retroviral entry possible [31].

2.1.1.4. Future directions. Major improvements in technology are required prior to any clinical application of targeted retroviral vectors being considered. Current limitations include both inefficient targeting and low titers of the targeted vectors. Genetic modifications can result in the inefficient production of functional env and/or in alterations in env activity. Production of a functional modified env needs to meet several requirements [7] such as: (1) proper control of translation and protein stability, (2) glycosylation, (3) oligomerization, (4) transport from endoplasmic reticulum to Golgi compartment, (5) transport to the cell surface, (6) incorporation into viral particles, (7) recognition of the viral receptor, and (8) control of the fusion of the viral envelope to the membrane of infected cells. Thus, it is not surprising that even limited genetic modifications can alter the balance between the different functional domains of the molecule and lead to inefficient targeting. An in-depth understanding of the quaternary structure and function of retroviral envelope is an important pre-requirement prior to targeting modifications being attempted. Retrovirus display libraries can improve targeting strategies by enhancing our ability to in vivo select targeting motifs [32]. Nevertheless, improvement of viral titers will be crucial prior to any translational effort involving retargeted retroviruses being initiated. Although pseudotyping can achieve that, in most cases, it expands rather than restricts viral tropism.

2.1.2. Retargeting of adenoviral vectors

Three distinct sequential steps are required for adenoviral infection. These include attachment of the adenovirus to a specific receptor on the surface of the target cell, internalization of the virus, and transfer of the viral genome to the nucleus where it can be expressed. Therefore, any effort to change the tropism of an adenoviral vector in order to be successful should preserve the adenovirus ability to perform these three functions. Two different cellular receptors are responsible for adenoviral entry, one mediating attachment and the other mediating internalization [33,34]. Binding of type 2 and 5 adenovirus to the primary cellular receptor, the coxsackievirus–adenovirus receptor (CAR), occurs through the knob domain of the fiber capsid protein, as demonstrated by the ability of the recombinant knob or anti-knob antibodies to inhibit adenoviral attachment [35,36]. After attachment, internalization of the virion occurs through receptor mediated endocytosis [37,38]. This process is mediated by the interaction of Arg–Gly–Asp (RGD) peptide sequences in the penton base with cellular integrin receptors. Hence adenoviral attachment and internalization are two distinct sequential steps. Following internalization, the virus is localized within the cellular vesicle system, initially in clathrin coated vesicles and then in cell endosomes [37,38]. Acidification of the endosomes allows the virions to escape and enter the cytosol. The virion then localizes to the nuclear pore and its genome is translocated to the nucleus of the host cell [38].

As it can be expected based on the steps described above, approaches to adenoviral vector targeting include use of bifunctional crosslinkers, structural modification of the fiber and modification of the penton base (Fig. 2).

2.1.2.1. Adenoviral vector targeting by use of bifunctional crosslinkers. In order to limit gene transfer exclusively

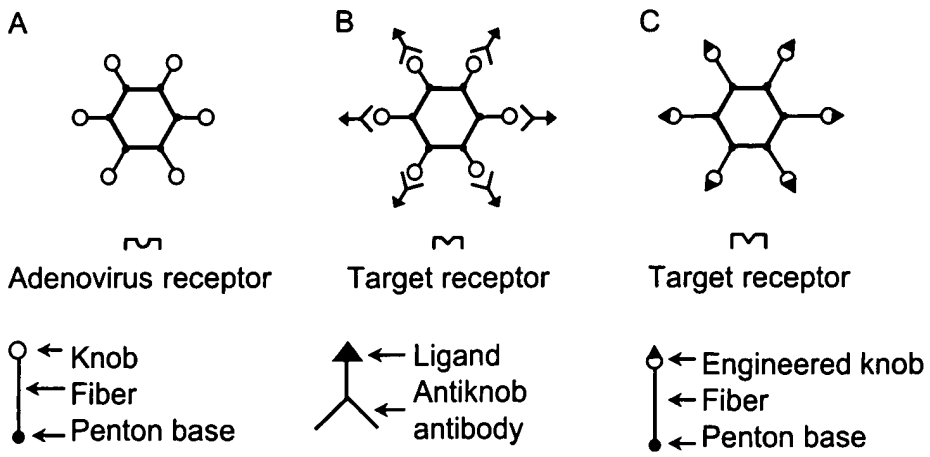

Fig. 2. Adenoviral targeting strategies. (A) Adenoviral entry through the natural viral receptor. (B) Use of bifunctional crosslinkers that recognize both the adenoviral knob and a target receptor in the cell surface can retarget adenoviral entry. (C) Engineered knob permits adenoviral entry through the target receptor.

to the target cancer cells, it is necessary to block the interaction between the knob domain of the adenovirus fiber and its cellular receptor, this interaction being a major determinant of adenoviral tropism. Anti-knob monoclonal antibodies would be capable of blocking the primary interaction between the adenovirus fiber and its cognate cellular receptor. In addition, retargeting can be achieved by employing anti-knob antibodies chemically conjugated to different ligands recognizing specific cell surface receptors. For example the Fab fragment of the anti-knob antibody was conjugated to folate in order to target the high affinity folate receptor which is overexpressed on the surface of several malignant cell lines, including ovarian, lung, and breast carcinomas [39]. Retargeting of an adenoviral vector carrying the luciferase reporter gene was achieved with levels of gene transfer comparable to that achieved by a native adenoviral infection in KB cells (human nasopharyngeal carcinoma cell line) [40].

Another effort of adenoviral retargeting by employing bifunctional crosslinkers includes generation of chemical conjugates between antiviral knob monoclonal Fab fragment and fibroblast growth factor (FGF2). The fibroblast growth factor receptor is a high affinity receptor overexpressed on a variety of tumor cells, including glioma cells and ovarian, pancreatic, and breast cancer cells [41]. This strategy has been employed in order to retarget adenoviral infection in different carcinoma cell lines including Kaposi sarcoma cells [42,43] as well as after intraperitoneal administration in an ovarian carcinoma model [44]. The same virus is currently undergoing phase I clinical testing.

Another family of receptors frequently amplified in human tumors, especially high-grade gliomas, is EGFR. By employing a bispecific antibody consisting of an antiknob monoclonal Fab fragment conjugated with an anti-EGFR monoclonal antibody, Miller et al. [45] achieved EGFR specific gene transfer and significantly enhanced adenoviral gene deliver in 7/12 established glioma cell lines. Watkins et al. [46] linked EGF to single chain Fv antibody fragments (ScFv) specific to the fiber. The EGF–ScFv fusion protein enabled targeted adenoviral gene delivery through EGFR.

A significant limitation with the bifunctional-crosslinker approach is related to the fact that the neutralizing antibody fragment is not covalently linked to the vector particle: the complex could, therefore, dissociate in the blood stream following intravenous administration.

2.1.2.2. Adenoviral vectors targeting by structural modification of the fiber protein. Another approach to adenoviral retargeting involves genetic engineering of the knob domain of the fiber protein. The introduction of heterologous cell targeting peptides, in the knob domain, requires consideration of the strict structural limitations of the fiber three-dimensional configuration. The fiber is synthesized as a monomer, which undergoes trimerization prior to its attachment to the penton base. Thus, it is necessary that any modification of the knob domain of the fiber does not impair trimer formation. In addition, the final quaternary configuration of the engineered fiber should make the incorporated ligand accessible to target cell receptor recognition and binding.

Recombinant adenoviruses have been constructed in which a heparin/heparan sulphate-binding domain, consisting of polylysine residues was added to the C-terminus of the fiber. Gene transfer to different mammalian cells including those that lack significant levels of the fiber receptor was achieved with a level of efficiency of 10–300-fold higher as compared to unmodified vector, the main drawback being lack of specificity since most mammalian cells express heparin containing cellular receptors [47].

Genetic modification of the C-terminus of the Ad fiber can be limited by the fact that the addition of larger (more than 25–30 amino acid residues) heterologous protein sequences can have a negative effect on the stability of the fiber trimer, which is incompatible with the fiber functions [48]. Recently, Dmitriev et al. [49] reported on the construction of modified adenoviral vectors containing the RGD (Arg–Gly–Asp) peptide in the HI loop rather than the C-terminus of the fiber knob domain. An increase in gene transfer to ovarian cancer cell lines (30–600-fold) and ovarian cancer cells obtained by patients (2–3-fold) was demonstrated by this vector. The greatest difference was seen with the ovarian cancer cell line OV-4 that expresses only modest levels of the CAR receptor, therefore suggesting that recombinant Ad containing fibers with an incorporated RGD peptide may be of significant utility for gene transfer in neoplasms characterized by deficiency of the primary Ad type 5 receptor.

2.1.2.3. The generation of targeted adenoviral vectors by modification of the penton base. Adenoviral retargeting efforts have also been focused on the modification of the penton base, which mediates the second step of adenoviral infection, i.e., internalization. Recombinant adenoviral vectors were generated in which the RGD motif in the penton base was substituted by the FLAG peptide. Complexing of this vector with a bispecific antibody consisting of a monoclonal antibody to the FLAG epitope and a monoclonal antibody to integrins resulted in the targeted infection of cells lacking the adenoviral fiber receptor such as endothelial cells or human intestinal smooth muscle cells. Thus, the first two steps of adenoviral infection binding and internalization were both mediated by A_V integrins [50]. Finally, recombinant adenoviruses were constructed containing chimeric penton base proteins that recognize tissue specific integrin receptors by replacing the wild-type RGD motif with avβ3 or a4β1 specific peptide motifs. The latter integrins are expressed at high levels on lymphocytes and monocytes but not on epithelial or endothelial cells, and recombinant adenoviruses based on this penton modification were successfully used to target lymphocytes [51].

2.1.2.4. Future directions. Most of the targeting strategies described above have exploited targeting moieties such as antibodies and growth factors directed to receptor molecules which are known to be expressed on the surface of the target tumor cells. However, many of these receptors are overexpressed not only in a number of carcinomas but are also expressed in normal cells, although in lower levels, which could limit their utility in targeting a particular subset of cancer cells. Thus, there is a need for ligands which possess a high degree of specificity for particular cancer cells. A possible solution to this problem is use of technology which permits the identification of cell specific ligands without requiring that the molecules against which they are targeted be identified. This technique involves the screening of random peptide presenting phage libraries for their ability to bind to specific cell types [52]. Another approach for target identification involves the ability of phage display libraries to target organs in vivo [53].

The role of targeting becomes more important when systemic gene therapy approaches (rather than intratumoral administration of transgenes) are contemplated. In this setting, identification of ideal ligands by itself cannot accomplish clinically meaningful targeting, until other problems associated with systemic adenoviral delivery are addressed. Examples of problems to be solved include systemic immune response against the adenoviruses, and the issue of stability of the adenovirus/bifunctional crosslinker complexes in the bloodstream. Ongoing clinical trials with hepatic artery or systemic adenoviral administration will help enhance our understanding of the adenoviral pharmacokinetics after single or repeated systemic administration and may contribute to a rational, clinically oriented design of adenoviral targeting strategies. Early results from clinical trials involving hepatic artery administration of adenoviruses indicate that large adenoviral particle loads ($>2\times10^{13}$) can lead to hypotension or life threatening multi-organ failure in certain clinical settings, such as the fatal outcome in a patient with ornithine-transcarbamylase deficiency [122]. In contrast, lower doses administered in the hepatic artery of patients with metastatic cancer to the liver appear to be safe and well tolerated. These results emphasize the importance of targeting in order to achieve the same clinical result, but with an improved safety profile, by employing lower titers of targeted vectors.

2.1.3. Adeno-associated virus targeting

The AAV is unique in its ability to target viral integration to a defined region of human chromosome 19. The specificity of integration is dependent on the presence of the inverted terminal repeats and the expression of the rep gene. Recombinant AAV lose their ability for site specific integration since the rep gene is deleted [54]. A chimeric AAV capsid protein was engineered, expressing the variable region of a single-chain antibody against the human CD34 molecule, a cell surface marker for hematopoietic stem/progenitor cells. Inclusion of the chimeric CD34 single-chain antibody-AAV capsid proteins within an rAAV virion significantly increased the preferential infectivity of rAAV for the CD34-positive human leukemia cell line KG-1, which is normally refractory to rAAV transduction [55]. Cell specific targeting was also achieved by a bispecific F (ab)2 antibody that allowed transduction of

human megakaryocytic cell lines by mediating AAV vector interaction with a cell surface receptor expressed on human megakaryocytes [56].

2.1.4. Sindbis virus targeting

Sindbis virus can infect a broad range of insect and vertebrate cell types due to the widespread distribution of its cellular receptor. The development of Sindbis virus vectors that target specific cell types is particularly important if the virus is to be used for the gene therapy of human malignancies. Sindbis virus particles were constructed displaying the IgG binding domain of protein A. This chimeric Sindbis virus vector had minimal infectivity against baby hamster kidney and human cell lines. However, when used in conjunction with monoclonal antibodies that react with cell surface antigens, the protein A envelope chimeric virus was able to infect human cell lines with high efficiency. Infection rates were 90% or higher for human lymphoblastoid cells [57]. Sindbis virus containing the HSV-tk gene and packaged in a helper virus displaying the IgG binding domain of protein A envelope could infect various tumor cell lines in the presence of bispecific antibodies that recognize antigens on their surfaces. HSV-tk transduced tumor cell lines exhibited sensitivity to ganciclovir (GCV) [58].

2.1.5. Herpes simplex virus 1 (HSV-1) targeting

Efforts for engineering of the HSV-1 envelope are currently in progress. An HSV-1 mutant virus deleted for glycoprotein C (gC) and the heparan sulfate binding domain of glycoprotein B (KgBpK-gC-) was engineered to encode different chimeric proteins composed of N-terminally truncated forms of gC and the full length erythropoietin hormone. Biochemical analysis demonstrated that one of the gC–EPO chimeric molecules was post-translationally processed, incorporated to recombinant HSV-1 virus and neutralized with antibodies directed against gC or EPO in a complement dependent manner. In addition, the recombinant virus was able to stimulate proliferation of EPO growth dependent cell line FD-EPO. However, the same cells were refractory to productive infection by both the wild-type and the modified virus. Electromicroscopy of FD-EPO cells showed that the modified virus was endocytosed and that resulted in an aborted infection. Despite the lack of productive infection, this data provided the first evidence of targeted HSV-1 binding to a non-HSV-1 cell surface receptor [59].

2.2. Transcriptional targeting

In order to approach current limitations in the ability to specifically target tumor cells for gene transfer, tissue specific promoters can be used that limit expression of the therapeutic gene to tumor cells and normal cells of a specific lineage. Alternatively, transcriptional regulatory sequences responsible for the expression of proteins preferentially produced in carcinoma cells such as oncogene products can be employed. Many tissue and tumor specific promoters have been developed [60,61], as seen in detail in Table 1. Tissue specific promoters have been used in gene therapy studies to evaluate tumor-specific killing mediated by expression of a suicide gene such as HSV-tk gene followed by exposure to GCV or cytosine deaminase gene followed by exposure to 5FC. For example, use of the albumin and alpha-fetoprotein (AFP) promoter in retroviral constructs encoding HSV-tk specifically killed hepatoma cell lines but had marginal activity in other tumor cells derived from breast, colon or skin [64]. Introduction of the murine tyrosinase promoter in a plasmid DNA encoding HSV-tk led to tumor regression after intratumoral inoculation in a B16 melanoma

Table 1
Tissue specific promoters

Promoter	Tissue/tumor type	References
Insulin promoter	B-islet cells	[60]
Whey acidic protein promoter	Breast	[61]
Tyrosinase promoter	Melanocytes	[62]
Glial fibrillary acidic protein promoter	Brain astrocytes, glioma cells	[63]
Albumin promoter	Liver	[64,65]
CEA promoter	GI tract, breast, lung	[66]
T-cell receptor promoter	T-lymphocytes	[67]
PSA promoter	Prostate	[68]
a-Fetoprotein (AFP) promoter	Hepatocellular carcinoma	[64]
Circulatory leukoprotease inhibitor (CLPI) promoter	Carcinomas, i.e. lung, colon, breast, oropharyngeal, bladder, ovarian, endometrial	[69]
Her 2/*neu* promoter	Breast, pancreatic, gastric carcinomas	[70,71]
Myc-Max responsive element promoter	Lung carcinoma	[72]
MUC-1 promoter	Adenocarcinomas	[73]
Osteocalcin promoter	Osteosarcoma	[74]

model. Melanocytes were targeted, but no local toxicity was observed in normal tissues adjacent to the tumor explant [62].

The CEA promoter has also been used to control transcription of the HSV-tk [66]. CEA expressing lung cancer cell lines were highly sensitive to GCV in vitro and in vivo following gene transfer of these constructs, whereas CEA non-expressing lung cell lines were resistant to GCV following gene transfer.

Another opportunity to specifically target tumor cells for gene expression is to use promoter elements that become activated in chemotherapy resistant tumor cells. Based on the observation that metallothionein promoter becomes activated in cisplatin-resistant ovarian carcinoma cells, plasmid DNA encoding the HSV-tk gene transcriptionally controlled by the metallothionein promoter has been introduced in cisplatin-sensitive and resistant ovarian carcinoma cell lines followed by treatment with GCV [75]. No cytotoxicity was apparent in cisplatin-sensitive parental 1A9 ovarian carcinoma cells, whereas cisplatin resistant subclones were efficiently killed by this treatment. This result suggests a specific approach for gene therapy of cisplatin-resistant ovarian carcinoma cells, and underscores the potential of using tumor specific promoter elements. Finally, transcriptional targeting has been employed in order to achieve condition targeted transgene expression. Examples include use of the radiation inducible elements of the early growth response-1 (egr-1) gene, a gene normally regulated by ionizing radiation [76–78] and use of hypoxia-responsive enhancer elements to achieve hypoxia-regulated gene expression [79].

One of the limitations of transcriptional targeting is variability in transgene expression, influenced by promoter activity, and frequently resulting in insufficient expression of the therapeutic transgene. One possible way to overcome this problem is use of the Cre/loxP system as it has been recently described for CEA-producing tumors [80]. A pair of recombinant adenoviruses was constructed, one expressing the recombinase (Cre) gene under the control of the CEA promoter (Ad.CEA-Cre) and the other containing the herpes simplex virus thymidine kinase (HSV-tk) gene separated by the strong CAG promoter by insertion of the neomycin resistance (neo) gene (Ad.lox-TK). The HSV-tk gene of the second adenovirus is designed to be activated through excisional deletion of the neogene by Cre enzyme, released from the first adenovirus, only when CEA producing cells are infected simultaneously with these adenoviruses. Co-infection by both adenoviruses made a CEA-producing cancer cell line 8.4-fold more sensitive to GCV as compared to infection with Ad.CEA-TK alone, the HSV-tk gene of the latter being directly regulated by the CEA promoter. Intratumoral injection of Ad.CEA-Cre combined with Ad.lox-TK followed by GCV almost completely eradicated subcutaneous CEA-producing tumors in nude mice while intratumoral injection of Ad.CEA-TK with GCV only retarded their growth [80].

3. Replication competent vectors

Use of non-replicating viruses or non-viral systems as vectors can limit the maximum achievable efficiency of gene transfer. In contrast, use of replicating vectors to allow replication of genes delivered initially to a small number of tumor cells and their subsequent transfer to neighboring cells, as the infection spreads, can significantly increase the efficacy of gene delivery.

As with live viral vaccines, replicating viral vectors raise serious safety concerns relating to the inherent mutagenicity of animal viruses. Prior to a replicating vector being tested in the clinic several safety considerations should be addressed [81].

- Can the replicating vector be pathogenic for the treated patients or damage normal tissues? If so, what would be the severity of the illness and is there an effective treatment available for it?
- Can the replicating vector undergo mutation or recombination in vivo to a new strain potentially more harmful than the replicating vector or its wild-type virus counterpart?
- Can the replicating vector or a more pathogenic mutant spread from the patient to other human subjects causing an epidemic? What are the possible modes of spread and how can they be controlled?
- Can the recombinant virus or a more pathogenic mutant cause harm to a developing embryo or fetus?

When selecting a virus from which a replicating vector for human use can develop, the potential for toxicity should be the primary consideration. Serious epidemics can arise when pathogenic viruses gain access to susceptible populations under conditions which favor transmission between individual members of the population [82]. For example, viruses which naturally occur in non-human species and are non-pathogenic for humans seem, at first sight, to be attractive candidates. However, when these viruses are adopted for replicative spread and in the absence of natural immunity, the entire human population could be susceptible to the new pathogen. A replicating vector for cancer therapy should, therefore, be derived from viruses, which are naturally endemic to the human population. However, serious human pathogens are obviously unsuitable because of the risk of reversion to a wild-type in the treated patients. The optimal strategy might be to derive a replicating vector from a highly prevalent but weakly pathogenic human virus. Reversion to wild-type would then be of no serious risk to the patient or to the population, but the possibility of recombination or a mutation giving rise to a pathogenic variant of the

original virus would have to be addressed. For example, it could be dangerous to extend the restricted tissue tropism of any virus by envelope protein engineering or promoter insertion. Conversely, restricting the tissue tropism of a virus already capable of replicating in a variety of human tissues would pose no serious risk.

The choice of a therapeutic gene for insertion into the replicating vector genome also has important public safety implications. A weak pathogenic replicating virus might be converted to a dangerous pathogen by insertion of gene coding for a toxin, whereas the gene coding for a drug activating enzyme would carry no such risk, since administration of the prodrug would be necessary for toxicity to occur.

The first efforts for treatment of cancer by employing replicating viruses go back to the 1950s and 1960s. After intraarterial or intratumoral administration of oncolytic viruses such as the mumps virus (paramyxovirus), transient tumor responses were seen. These early studies were limited by the inefficient virus production, purification and concentration techniques available at that time [83,84]. In the last decade, the interest in replicating viruses in gene therapy of cancer has resurfaced. Most of the work both in pre-clinical models and clinical trials focuses on adenoviruses and herpes viruses since both classes of viruses meet some of the criteria described above. Other viruses with potential interest in this setting include reoviruses and the Newcastle disease virus (NDV), the latter being used mainly in ex-vivo approaches.

3.1. Replicating adenoviruses

Type 5 adenoviruses meet several of the criteria described above [85]. Adenovirus type 5 is already prevalent in the human population, is of low pathogenicity, does not persist in the infected host, and there is a safe, effective vaccine available. Disadvantages include the presence of anti-adenovirus neutralizing antibodies in approx. 80% of the patients, which can negatively affect systemic delivery. Two main approaches have been followed in order to specifically target replication competent type 5 adenoviruses against tumor cells: (a) deletion of the E1B region that allows viral replication only in cells with malfunctioning p53, and (b) use of tissue specific promoters to control adenoviral replication.

3.1.1. Deletion of the E1B region

The 55 kDa protein encoded by the E1B region of an adenovirus binds and inactivates p53. Because p53 function must be blocked in order to allow efficient adenoviral replication, an adenovirus attenuated in the E1B region (such as the ONYX-015 adenovirus) is limited in its replication in normal cells; however, cancer cells with malfunctioning p53 should support virus replication and cell lysis [86]. The p53 is mutated in roughly 50% of all human cancers, including non-small cell lung cancer (60%), colon (50%), breast (40%), head and neck (60%), and ovarian cancer (60%). Several recent reports indicate that ONYX-015 replication in tumor cells can be independent of the p53 status [87,88]. In addition to p53 mutation however, functional inactivation of the p53 as caused by the MDM2 protein (product of the mdm2 oncogene) or the $p14^{ARF}$ protein can make the tumor cells permissive to viral replication. Finally, the E6 human papilloma virus protein can render cells susceptible to the ONYX-015 replication by blocking p53.

In nude mouse models, antitumor efficacy of ONYX-015 plus chemotherapy (cisplatin and 5-FU) was significantly greater than with either agent alone [89]. Interestingly, development of cisplatin and doxorubicin resistance in A2780 ovarian carcinoma cell lines made the cell susceptible to ONYX-015 replication despite the wild-type p53 status [90].

In phase I trials, intratumoral administration of ONYX-015 in doses up to 10^{11} pfu and different repeated administration schedules proved to be safe and well tolerated in different tumor types including head and neck [91], pancreas [92], GI metastatic to the liver [93], as well as ovarian cancer [94] (intraperitoneal administration). The intratumoral administration of ONYX-015 in a daily × 5 schedule, concurrently with 5-FU and cisplatin chemotherapy (days 1–5: virus, days 1–5: 5-FU, and day 1 cisplatin) led to objective clinical responses in 63% of 30 evaluable patients with head and neck tumors, recurrent despite surgery and radiation [95]. There was no correlation between response and baseline tumor size, baseline neutralizing antibody titer, or p53 status. However, other molecular alterations such as mdm2 amplification that could lead in p53 malfunction despite the presence of a wild-type p53, were not examined in tumor specimens. The results of this trial, although very intriguing, will have to be confirmed in a larger randomized study. A phase III trial comparing ONYX-015/5-FU/CDDP to 5-FU/CDDP in patients with recurrent head and neck tumors, who have failed prior radiation therapy, is currently ongoing in the United States.

Two other clinical trials exploring the safety of systemic ONYX-015 administration were recently completed: the first study was a phase I/II dose escalation trial of ONYX-015 infused into the hepatic artery as a single agent and in combination with intravenous 5-FU/leucovorin in patients with liver predominant metastatic gastrointestinal carcinoma [120]. Viral doses up to 10^{11} pfu (2×10^{12} viral particles) were well tolerated. No clinically significant hepatotoxicity was observed. Pharmacokinetic analysis was suggestive of ongoing viral replication since a second peak in viral titers was observed at 3 days post-infusion. Antitumor

activity was observed [120]. The second trial was a phase I dose escalation study of intravenous administration with and without chemotherapy in patients with advanced malignancy. No dose limiting toxicity was identified in doses up to 10^{12} pfu. Selective viral replication in tumor tissue has been demonstrated in a metastatic lung lesion (John Nemunitis, personal communication). ONYX-015 has recently completed clinical testing in patients with pancreatic cancer (endoscopic intratumoral administration), and it is currently in clinical trials in patients with metastatic colon cancer who have failed prior chemotherapy (intravenous administration), patients with recurrent gliomas (intratumoral administration), and in recurrent sarcoma patients in combination with chemotherapy.

3.1.2. Utilization of tissue specific promoters to restrict adenoviral replication in specific tissues

Prostate specific antigen (PSA) is a widely used marker for the diagnosis and management of prostate cancer. Enhancer/promoter constructs derived from the 5′ regions flanking the human PSA gene (prostate specific enhancer) were inserted into adenovirus type 5 DNA prior to the E1A gene, thereby creating CN706, a prostate specific enhancer-containing provisionally replicating adenovirus. E1A was expressed at high levels in modified virus-infected human PSA producing LNCaP cells, but not in DU145 cells (human prostate cells that do not express PSA). Viral titer was significantly higher in LNCaP cells compared to several human cell lines that do not produce PSA. The modified type 5 virus destroyed large LNCaP tumors (1×10^9 cells) and abolished PSA production in nude mouse xenograft models with a single intratumoral injection [96]. A phase I trial of CN706 has recently been completed in patients with locally recurrent adenocarcinoma of the prostate. Up to 10^{13} viral particles were administered into the prostate using brachytherapy techniques. Treatment was well tolerated and some patients achieved biochemical (PSA) responses [121]. A similar virus, but with enhancer promoter constructs derived from the 5′ region of the human CEA, has also been constructed. Finally, provisionally replicating adenoviruses containing the AFP promoter [123] and the DF3/MUC1 promoter [124] have been shown to have antitumor activity against hepatocellular carcinoma and MUC-1 expressing tumor xenografts, respectively.

3.2. Replicating viruses of the herpes group

3.2.1. Herpes simplex virus type 1 (HSV 1)

HSV-1 is a natural pathogen in humans that can cause recurrent oropharyngeal cold sores. The virus is transmitted by close personal contact with an infected individual; initially the virus establishes a productive infection in epithelial cells, gains access to the sensory nerve ending supplying the infected area of skin, and travels by a retrograde axonal flow to neuronal cell bodies within the dorsal root ganglia [97]. Viral replication in the nervous system is generally limited and the virus can establish a latent infection within sensory neurons. During latency which lasts for the lifespan of the individual, the viral genome persists within the neural nucleus in the absence of detectable viral protein expression [98]. The lytic pathway of HSV infection in tissue culture cells has been extensively studied [99]. The viral genome consists of a long and a short unique region, each flanked by inverted repeats. Upon uncoating of the viral DNA in the nucleus of the infected cells, immediate early gene expression is activated by the action of the structural component of the viral particle Vmw65 (also known as VP16 or αTIF). A number of immediate early proteins function to activate early viral gene expression which, in turn, leads to replication of viral DNA followed by expression of the late, mostly structural, genes and the formation of the progeny virions. A number of genes required for virus neurovirulence are known to be non-essential in tissue culture and can be deleted. These include thymidine kinase, γ34.5 and ribonucleotide reductase. Pre-clinical data have indicated that herpes simplex virus vectors may be good candidates for treatment of brain tumors [100].

Martuza et al. developed a novel therapeutic approach using genetically engineered replication competent HSV-1 [100]. The first generation of HSV-1 vectors contained mutations in a single gene that restricted their replication to dividing cells. Three such HSV-1 mutants were constructed: (1) *dls*ptk containing a deletion in the thymidine kinase gene [100,101]; (2) hrR3 containing an insertion of the *Escherichia coli* lac-Z gene in the early gene ICP6 encoding the large subunit of HSV ribonucleotide reductase [102,103]; ribonucleotide reductase is a key enzyme for viral DNA synthesis required for efficient viral growth in non-dividing cells, but not in many dividing cells and tissue culture; and (3) R3616 containing 1-kb deletions in both copies of the γ34.5 gene, a gene responsible for neurovirulence [104].

In considering possible clinical trials with a replication competent herpes virus, it was essential that adequate safeguards were employed. In the second generation vectors, multigene mutants of HSV-1 were created with deletions in both γ34.5 loci and a lac-Z gene insertion in the ICP6 gene [105,106]. These multiple mutations made the reversion to wild-type highly unlikely and conferred several important safety advantages. When this double mutant G207 was administered intratumorally in nude mice harboring subcutaneous or intracerebral gliomas, it caused decreased tumor growth and/or prolonged survival. G207 was avirulent upon intracerebral inoculation in HSV-sensitive monkeys. The results of a phase I clinical trial in 21 patients with

recurrent high-grade gliomas have been recently reported [107]. No serious toxicity, which could be unequivocally attributed to G207, was seen in doses up to 3×10^9 pfu, and no patient developed HSV encephalitis. MRI tumor volumetrics were used to assess antitumor response. Although none of the patients met standard oncologic criteria for objective response, six patients had a decrease in the enhancement volume between the pre-inoculation scan and the 1-month post-inoculation MRI. The investigators were not able to prove in vivo replication of the virus. However, the tissues examined were derived from resection of progressive disease or autopsy at least 60 days after inoculation. In two patients, HSV-1 and lac-Z sequences were detected by PCR, indicating that G207 DNA was present in the specimen.

Ongoing work aims at modification of mutant replication competent HSV-1 by adding cytokine genes such as IL-4 or IL-10 which can increase tumor immune recognition [108]. In another approach, insertion of the p450 gene allows the activation of cyclophosphamide in infected cells [109]. Finally, cell specific promoters (such as albumin, expressed in hepatocellular carcinoma) have been used to target replication of HSV-1 [110].

3.2.2. Epstein-Barr virus (EBV)

The presence of Epstein-Barr virus (EBV) genome in certain tumor types (in particular nasopharyngeal carcinomas and AIDS-related CNS lymphomas) may allow novel EBV-based targeting strategies. Tumors contain the latent form of EBV. However, expression of either of the EBV immediate early proteins BZLF1 and BRLF1 is sufficient to induce lytic EBV infection resulting in death of the whole cells. Westphal et al. [111] were able to induce lytic EBV infection in an EBV positive Burkitt lymphoma cell line by employing replication deficient adenovirus vectors expressing the BZLF1 or BRLF1 immediate early gene. Furthermore, lytic EBV infection converts the antiviral drug ganiciclovir into the toxic (phosphorylated form) which inhibits the cellular as well as viral DNA polymerase. In this way, while viral reactivation is induced and the Burkitt lymphoma cells are efficiently killed, the release of infectious EBV particles can be prevented with use of ganciclovir [111].

3.3. Reovirus

The human reovirus is a non-enveloped virus containing double-stranded RNA. Reovirus infections in humans are believed to be mild and restricted to the upper respiratory and gastrointestinal tract. The virus replicates employing an activated ras signaling pathway. Restriction of reovirus replication in untransformed NIH 3T3 cells is due to the activation of the double-stranded activated protein kinase (PKR) by early viral transcripts which, in turn, inhibits the translation of these transcripts. Activated ras or an activated element of the ras pathway presumably inhibits (or reverses) PKR activation, thereby allowing viral protein synthesis. Of note, activating mutations of the proto-oncogene ras occur in about 30% of human tumors, primarily in pancreatic (90%), sporadic colorectal (50%) and lung (40%) carcinoma and myeloid leukemia (30%) [112]. The selective replication of reovirus in cells with an inactivated ras signaling pathway in combination with the relatively non-pathogenic nature of this virus in humans [113,114] makes it attractive as a potential oncolytic agent. SCID mice bearing tumors established from v-erb-B transformed murine NIH 3T3 cells or human U87 glioblastoma cells were treated with the virus. Both cell lines have increased levels of activated ras. A single intratumoral injection of virus resulted in tumor regression in 65–80% of the mice. Although reovirus is not associated with any major human diseases, 50–60% of reovirus-treated SCID mice eventually developed lethal hind limp necrosis. The immunodeficiency of these animals was probably responsible for the observed high mortality rate. Treatment of immunocompetent C3H mice bearing tumors established from ras transformed C3H 10T1/2 cells also resulted in tumor regression although multiple injections were required as a result of a competent immune system. Side effects however, were also alleviated [115]. The human reovirus is soon to be taken to phase I/II clinical trials for patients with advanced lung, breast, and colon cancer.

3.4. Newcastle disease virus (NDV)

The majority of the gene therapy applications employing replicating viruses are directed to in vivo gene therapy of cancer. In contrast, non-virulent strains of the avian paramyxovirus, NDV, have been employed for ex vivo infection of tumor cells and generation of more effective autologous tumor vaccines [116,117]. NDV has pleiotropic immune stimulatory properties in addition to good cell-binding and selective proliferation in replicating cells. Most important for its use as an adjuvant in human cancer vaccines is its ability to introduce T-cell co-stimulatory activity and induce cytokines such as IFN-a, IFN-b and TNF-a that affect T-cell recruitment and activation [117]. In a modified approach, following the infection of tumor cells with a non-virulent strain of NDV, the cells are co-incubated with bispecific antibodies which recognize both the viral hemagglutinin–neuraminidase molecule on the infected tumor cells and CD28 activating epitopes on T-lymphocytes [118]. Autologous live cell NDV modified tumor vaccines (ATV–NDV) were administered in a phase II setting for different tumor types including breast and colon. A prospective randomized phase III

study of adjuvant ATV–NDV administration for breast cancer patients is currently underway in Europe [119].

In summary, provisionally replicating viruses are gradually establishing their presence in cancer gene therapy clinical trials as single agents, vectors, or in combination with chemotherapy. The balance between virulence and therapeutic efficacy will always have to be carefully considered as dose finding studies continue and new agents enter human clinical trials. Targeted replicating viruses may represent the ideal answer to the gene delivery problem, given the systemic nature of most human malignancies.

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Biographies

Evanthia Galanis, M.D. received her M.D. degree from Athens University Medical School, Athens, Greece. She is a Senior Associate Consultant in Oncology and Molecular Medicine, Mayo Clinic, Rochester, MN.

Richard Vile, Ph.D. received his Ph.D. from the University of London, England. He is a Consultant in Molecular Medicine, Mayo Clinic, Rochester, MN.

Stephen J. Russell, M.D., Ph.D. received his M.D. from Edinburgh University and his Ph.D. from the University of London, England. He is a Consultant and Director of the Molecular Medicine Program, Mayo Clinic, Rochester, MN.

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TARGETED, NON-VIRAL GENE DELIVERY FOR CANCER GENE THERAPY

Richard J. Cristiano

Section of Thoracic Molecular Oncology, Department of Thoracic and Cardiovascular Surgery, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Box 109, Houston, Texas 77030

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1. ABSTRACT

The ability to mediate targeted and specific delivery of therapeutics to cancer cells remains one of the most important hurdles in effectively treating cancer. This aspect also remains as one of the greatest limitations of gene therapy as well. Targeted vectors based on the use of DNA-binding agents attached to cell specific ligands or "molecular conjugates" were created with the goal of over-coming this hurdle. Since being conceived, many different ligands have been utilized as molecular conjugates, targeting the resulting Protein/DNA polyplex to cells efficiently *in vitro* while mediating limited delivery in vivo. This limited delivery is due to many reasons such as the need to identify non-viral agents that can aide in escaping endosome entrapment as well as decreasing the complexity that has evolved in the creation of these "synthetic viruses". This review will discuss the current status and the future of molecular conjugates as targeting vectors as well as the positive and negative attributes of this vector in relation to other viral and non-viral vectors that are currently used in many gene therapy strategies.

2. INTRODUCTION

The use of gene therapy for the treatment of diseases of both genetic and infectious origins has now become a reality. Crucial to the success of any gene therapy strategy is the efficiency with which the gene is delivered. This in turn is dependent upon the type of delivery vector used. Many vectors have been developed based on either recombinant viruses or non-viral vectors (1). Because of the highly evolved components of viruses, research utilizing these vectors has progressed much more rapidly than non-viral vector development. This is reflected by the fact that approximately 85% of current clinical protocols involving gene therapy utilize vectors based on viruses (2). Unfortunately, these vectors are still limited in many ways, particularly in relation to issues of safety, immunogenicity, limitations on the size of the gene that can be delivered, specificity, production problems, toxicity, cost and others.

Crucial to the development of any delivery vector is the need for the following characteristics: 1) the capability of targeting specific cells; 2) no limitation on the size or the type of nucleic acid that can be delivered; 3) no intact viral component for virus reproduction and therefore safe for the recipient; 4) the ability to transduce a large number of cells regardless of the mitotic status; and 5) the potential to be completely synthetic. Although this list is not inclusive and is not required for all vectors in every application, it does provide some basic criteria that are required for vector construction. The most important aspect of any vector is that of specificity. This is particularly important in relation to cancer gene therapy where the goal is either to mediate growth arrest and/or apoptosis specifically in the tumor cell and not in normal cells.

As a step toward the development of a non-viral, targeted gene delivery vector, molecular conjugates have been created. Molecular conjugates are ligands to which a nucleic acid or DNA-binding agent has been attached with the specific goal of targeting nucleic acids (e.g. plasmid DNA) to cells. When combined with DNA, the resulting Protein/DNA polyplex (termed polyplex based on the use of polycations as a DNA binding agent) can consist of at least four components: 1) a ligand for cell-specific targeting; 2) a nucleic acid or DNA-binding agent (that is chemically attached to the ligand) for the binding of the DNA by ionic or other non-damaging interactions; 3) a nucleic acid i.e., a plasmid for gene expression; and 4) an endosomal lysis agent to enhance the release of the nucleic acid from the endosomal compartment into the cytoplasm of the cell (figure 1). The goal of this review is to explain the rationale for using molecular conjugates as a delivery vector for cancer gene therapy as well

Table 1. Ligands used as molecular conjugates for targeted DNA delivery and current disease applications

LIGAND	TARGET CELL/ORGAN	DISEASE
Asialoorosomucoid	Hepatocyte/liver	Analbuminemia, Phenylketonuria,
Transferrin	Liver, lung, and many others	Melanoma, And many other applications
Folate	Cancer cells; KB, Hela	Ovarian cancer
Adenovirus fiber	Lung, liver, many others	Lung cancer, Phenylketonuria,
Malaria cs protein	Hepatocyte/liver	Hepatocellular Carcinoma, Diabetes,
Epidermal growth Factor	Lung, brain, and Pancreatic cancer cells, other	Lung, brain, pancreatic and other types
Human papilloma Virus capsid	Epithelial cells/cervix	Cervical cancer
Fibroblast growth Factor	Brain	Brain cancer

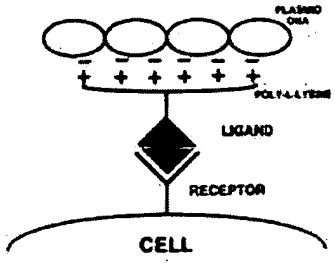

Figure 1. The general structure of a targeted DNA delivery vector. The ligand, which has receptors on a specific cell type, is modified to allow for a covalent interaction with a DNA-binding agent, such as poly-L-lysine, to form a molecular conjugate. The positive charge from the amino groups of the poly-L-lysine interact with the negative charge from the phosphate groups on the DNA, allowing for a non-damaging ionic interaction.

as discussing the formation of Protein/DNA polyplexes, the components involved, present and future applications, and the advantages and disadvantages of this vector in relation to other non-viral and viral gene delivery vectors.

3. CELL-SPECIFIC TARGETING LIGAND

Molecular conjugates were originally created as a non-viral gene delivery vector that maintains the basic characteristics of a virus (the ability to transport and deliver nucleic acids into cells), while being able to target therapeutic genes to specific cell types. The targeting is accomplished through the use of ligands that have receptors expressed on a specific population of cells. An example is the glycoprotein asialoorosomucoid (ASOR) which is specifically endocytosed by the liver parenchyma, since the receptor for this ligand is expressed almost exclusively on these cells (table 1) (3).

Asialoorosomucoid was first utilized as a molecular conjugate by Wu *et al.*, for targeting DNA into liver cells (3). In this experiment, a plasmid expressing the chloramphenicol acetyl-transferase (CAT) reporter gene was delivered by an ASOR conjugate into HepG2 cells (a liver cell line that expresses 250,000 ASOR receptors/cell). The cells exhibited transient, low level CAT expression that could be competed with free ASOR, indicating cell-specific targeting. Further analysis in mice, utilizing a tail vein injection of the ASOR/DNA polyplex resulted in liver specific expression of CAT (4). While this represents one of the first attempts to use a ligand as a molecular conjugate for gene delivery, many other types of ligands have now been utilized (table 1).

Transferrin, which has receptors that are expressed by many different cell types, has been used as a molecular conjugate to deliver DNA to erythroleukemic, lung, and liver cell lines (5). The targeted delivery of DNA to cells via the transferrin receptor also provides an example in which the status of a receptor can be modulated to result in higher levels of gene delivery. Agents such as desferrioxamine, an iron chelator, can result in a 4-fold increase in the number of receptors on responsive cells and thus lead to increased cell transduction by a Transferrin/DNA polyplex (6). Smaller ligands such as the vitamin folate have also been used as a molecular conjugate to promote delivery of DNA into cells that over-express the folate receptor such as on ovarian carcinoma cells (7). More recently, other ligands have been used to promote selective or specific uptake by certain cell types that vary in receptor expression. The over-expression of receptors for epidermal growth factor (EGF) on cancer cells has allowed for specific uptake of EGF/DNA polyplexes by lung cancer cells (8). The malaria circumsporozite (MCS) protein has been used for the liver-specific delivery of a MCS/DNA polyplex during conditions in which ASOR receptor expression on hepatocytes is low, such as in cirrhosis, diabetes, and hepatocellular carcinoma (9). More recently, other growth factors have been used for targeting nucleic acids to cells such as fibroblast growth factor and its receptor (10). Even more complex protein structures such as viral capsids have also been used as molecular conjugates. The Human Papilloma virus (HPV) capsid was used by Muller *et al.* to partially identify the HPV receptor. This

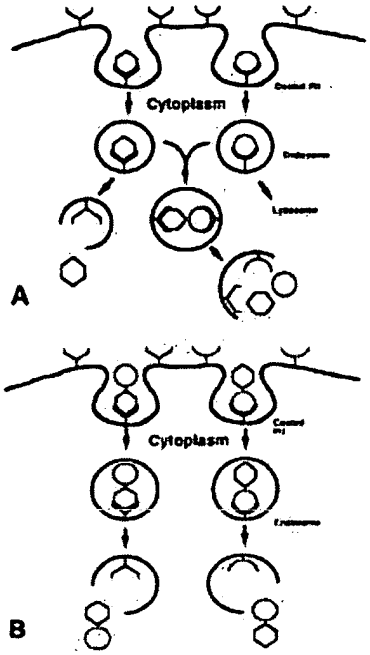

Figure 2. Uptake of a Protein/DNA polyplex or a Protein/DNA/adenovirus polyplex into cells. (A) Coincubation of the adenovirus with the Protein/DNA polyplex results in binding and uptake of each component through the associated receptor and requires cointernalization into the same endosome for endosomal lysis and release of the Protein/DNA polyplex. (B) Coupling of the adenovirus to the Protein/DNA polyplex results in the adenovirus accompanying the Protein/DNA polyplex into the same endosome for endosomal release. As a result, the efficiency of gene delivery enhancement is increased, however, uptake through the adenovirus receptor is also possible. Circles represent the Protein/DNA polyplex; hexagons represent the adenovirus. (figures taken from Reference 21).

involved using the capsid as a ligand for the attachment and delivery of a reporter gene to cells (11). More recently, replication defective adenovirus was used as a DNA carrier that resulted in efficient gene delivery into malignant cells both *in vitro* and *in vivo* (12,13).

The continued identification of ligands for targeted gene delivery has also resulted in attempts to switch to synthetic ligands. Studies by Plank *et al.* identified that the protein ASOR, which binds to the ASOR receptor through terminal galactose groups, could be replaced by a galactose conjugate and still mediate liver specific DNA delivery (14). This conversion represents an important finding in that molecular conjugates can potentially be synthetically derived. Recent work has also shown that phage expression libraries can be used as a source for identifying unique ligands (15). As a result, the combination of further research and the simplicity of this system should allow for the identification and use of many other ligands for cell specific targeting to any cell of choice.

4. ENDOSOMAL LYSIS AGENTS; VIRUSES VS. PEPTIDES VS. POLYCATIONS

The utilization of ligands as molecular conjugates for targeting a DNA polyplex to specific cells, results in the passage of the polyplex into the cell by pathways of normal ligand/receptor internalization. One such pathway is based on receptor-mediated endocytosis and follows the general steps of: 1) binding of the ligand by its receptor; 2) internalization and endosome formation; 3) fusion with lysosomes; and 4) degradation of the contents of the endosome by lysosomal enzymes. In some instances, recirculation of the receptor back to the cell surface does occur (16). Overall, the outcome is that the DNA attached to a molecular conjugate becomes degraded along with the ligand, resulting in transient and low level gene expression (3,5). In the case of the folate receptor, the bound ligand is internalized through a process termed potocytosis, where the receptor binds the ligand, the surrounding membrane closes off from the cell surface, and the internalized material then passes through the vesicular membrane into the cytoplasm (7). As a result, the folate conjugate and attached DNA are not degraded but essentially remain trapped inside larger vesicles or "potosomes" in the cell as the Folate/DNA polyplex is unable to pass through the vesicular membrane.

Thus far, the ability to efficiently escape or bypass endosome or vesicle entrapment, remains one of the major limitations of this vector. However, the release of DNA into the cytoplasm of the cell can be enhanced by agents that either mediate endosome disruption, decreased DNA degradation, or bypass this process all together. Chloroquine, which raises the endosomal pH, has been used to decrease the degradation of endocytosed material by inhibiting lysosomal hydrolytic enzymes (5). Physical procedures, such as a partial hepatectomy, when performed along with DNA delivery by an ASOR/DNA polyplex, has resulted in increased persistence and expression of delivered DNA, from days to months (17). However, these procedures have been limited in use because of the lack of utility for enhancing endosomal release in other tissues. To overcome this limitation, Curiel *et al.* demonstrated that a human replication-defective adenovirus (i.e. dl312, serotype 5 adenovirus deleted of the E1a gene) could be utilized as an endosomal lysis agent (18). The adenovirus is internalized by a receptor-mediated process and escapes degradation by fusion of the viral capsid with the endosomal membrane, which results in membrane pore formation and leads to lysis of the endosome (19).

When replication defective adenovirus is combined with a Protein/DNA polyplex and co-incubated with cells, both are internalized into the same endosome, allowing for the adenovirus to mediate endosomal lysis which leads to the release of the DNA (figure 2A). The resulting level of gene expression can be increased 1000 to 2000-fold as demonstrated in reports where ASOR/DNA or Transferrin/DNA polyplexes were incubated along with the replication defective adenovirus dl312 (18,20). Unfortunately, viral titers of at least $1x10^3$ to $1x10^4$ viral particles/cell must be used, which can result in toxicity as well as raising questions of the specificity of delivery. Fortunately, the specificity of delivery is still maintained by the ligand in the Protein/DNA polyplex, as competition with free protein, results in a decrease in DNA delivery into the cells (18,20). Enhanced endosomal release of polyplexes by adenovirus has also been identified to work for several other ligands as well (table 1) (7-11).

Although high level transduction can be achieved *in vitro*, this system would not be practical for *in vivo* use. To create a more suitable polyplex for *in vivo* DNA delivery, the adenovirus has also been directly coupled to the Protein/DNA polyplex (figure 2B) (21-23). When this is done, at least a one-order of magnitude drop in the viral titer used and an increase in the specificity of endosomal lysis is achieved. This is due to an increase in the chance that an endocytotic event will generally be accompanied by an adenoviral particle. Unfortunately, this type of polyplex has shown limited use *in vivo*, due to problems such as non-specific uptake through the adenovirus receptor, an increase in the size of the polyplex, and increased toxicity (24). However, recent work by Nguyen *et al.* has shown that the core components of the vector i.e. the adenoviral particle, the polycation, and the DNA can be combined to mediate efficient delivery both *in vitro* and *in vivo* at levels sufficient to mediate inhibition of tumor growth through tumor suppressor p53 expression (12,13). although viral toxicity remains a problem, this can be decreased by using ultraviolet light to inactivate the viral genome (25). Other viruses have been used to mediate endosomal lysis as well, such as the chicken adenovirus (CELO Virus) (26). This virus is capable of pH-dependent endosomal lysis like the human adenovirus and can mediate similar levels of delivery enhancement, without the associated toxicity, however, aspects such as increased size and complexity remain a problem with this vector configuration.

To achieve a completely non-viral delivery vector and thus remove many of the problems associated with the presence of the virus, studies have also focused on using non-viral endosomal lysis agents. Peptides based on the membrane lysis portion of the influenza virus hemagglutinin HA2 have been incorporated into a Transferrin/DNA polyplex to promote endosomal lysis (27). As a result of endosomal acidification prior to fusion with the lysosome, these short, 20 amino acid peptides mediate endosomal lysis by inserting into the endosomal membrane, causing pores to form, which leads to lysis (28). Unfortunately, a comparison of these peptides to adenovirus has shown that they are less efficient at this process (27). As a result, there continues to be a focus on identifying agents that are capable of mediating this function as well as being capable of mediating multiple functions associated with polyplex formation and delivery, thus reducing vector complexity and size.

Recently, synthetic polycations have been the focus of developing such agents. While linear versions of polycations such as poly-L-lysine (PLL) are capable of mediating DNA compaction, this molecule is incapable of mediating endosome release (29). However, branched chain versions of polycations such as Polyethylenimine and Starburst dendrimers can mediate both functions (30). Polyethylenimine (PEI) is a highly branched polymer with a ratio of 1:2:1 of primary: secondary: tertiary amines. This compound combines the ability of DNA binding (and therefore the coupling of proteins and peptides to the vector) with the ability to mediate endosomal release through its ability to act as a "proton sponge". The polycation has terminal amines that are ionizable at pH 6.9 and internal amines that are ionizable at pH 3.9 and because of this organization, can generate a change in vesicle pH that leads to vesicle swelling and eventually, release from endosome entrapment (30). The ability of this agent to deliver genes has been demonstrated previously; the luciferase activity of a luciferase expressing plasmid in polyplex form with PEI was found to be comparable to "Transfectam" liposomes, at approximately 5×10^7 light units in murine 3T3 fibroblasts and significantly higher than 5×10^3 light units for poly-L-lysine (30). This polymer has also been used to transduce at least 25 cell lines and primary cells which resulted in high level transduction over a 5 order of magnitude efficacy range (31). The *in vivo* efficiency of this gene delivery vector has also been shown as well (30,32,33). Bousiff *et. al.* have shown that high level transduction of either plasmid DNA or oligonucleotides with low toxicity can be achieved in the brain at levels comparable to *in vitro* transduction efficiencies (30). Similar results were achieved by Boletta *et. al.* in which high level transduction was achieved in the kidney (33).

In contrast, a completely different approach has been chosen by Wels and associates (34). The focus is to completely bypass endosomal degradation by using proteins that utilize other pathways in cells to deliver nucleic acids into the nucleus. In this approach, subunits of toxins such as Diptheria toxin and Pseudomonas exotoxin have been utilized as components of chimeric proteins that can be incorporated into the polyplex (34). When these components are used, shuttling of the nucleic acid through the endosomal membrane

and back through the endoplasmic reticulum occurs (34). While nuclear delivery can be obtained, the resulting vector is still limited in size, complexity, and immunogenicity. However, further research in this area of utilizing proteins to bypass endosomal entrapment may lead to further advancements in enhancing gene delivery.

In general, a similar theme is occurring in the development of endosomal lysis agents, that has occurred for ligand identification in that complex proteins are being replaced with smaller functional subunits or simple, synthetic lysis agents. Overall, although replication-defective adenovirus is still the best endosomal lysis agent available, it is clear that this agent must be replaced to allow for the greater utilization of this vector, particularly *in vivo*.

5. DNA-BINDING AGENTS

Once a ligand or endosomal lysis agent has been identified for use in a Protein/DNA polyplex, the component must be modified to allow for attachment to DNA. As a result, the primary function of the DNA-binding agent is to bind DNA in a non-damaging interaction, resulting in attachment of the protein or peptide to the polyplex. Poly-L-lysine (PLL), which is a synthetic poly-cation that consists of repeating lysine residues, has been the most utilized agent thus far. This compound can be synthesized in various sizes ranging from 15 to over 1000 lysine residues in length. Molecular conjugates have been synthesized with PLL's of 15, 100, 250, and over 1,000 lysine residues (3-14,17,18,20-27). The binding of the DNA to the PLL occurs between the positive charge of the amino groups and the negative charge of the phosphate groups on the DNA. This contributes to complete charge neutralization on the DNA molecule which can be viewed by agarose gel electrophoresis as DNA that fails to migrate out of the well of the gel (3,20). During this interaction, the structure of the DNA molecule changes from a supercoiled or open circle form to a toroid structure (20,29). These structures can be viewed by electron microscopy after negative staining and are approximately 80-100 nm in size. This small size has been shown to contribute to efficient DNA delivery in many cell types, as the average size of the endosomal compartment is 100-200nm (20,29).

Unfortunately, the complexity of the resulting Protein/DNA polyplex and a lack of understanding its formation are persistent limitations. A recent report by Xu *et al.* has shown that the size of the DNA-binding agent PLL can have a great affect on particle size, charge, and gene delivery (35). In this study, smaller (80 nm) and more stable polyplexes were obtained with PLL of chain length's greater than 1000 than with shorter versions of PLL, especially in 0.15M NaCl. Stability was increased by adding streptavidin to the polyplex, however, the targeting ligand EGF increased particle size (>1000 nm) and decreased gene delivery when >300 EGF molecules per polyplex was used, indicating that a critical number of EGF molecules were needed for efficient gene delivery. The correct combination of these components resulted in the most efficient gene delivery *in vitro* and potentially *in vivo*.

Other naturally occurring DNA-binding agents, such as spermine or spermidine, have also been utilized for Protein/DNA polyplex formation (36). These proteins tend to have a lower binding affinity for the DNA which is due to their small size. At this point, it is unclear as to how tightly these agents must bind the DNA to contribute to efficient uptake and release of the DNA once inside the cell. Another class of DNA-binding agents, histone proteins, have also been modified for Protein/DNA polyplex formation (37). In this example, the proteins were modified by galactosylation for targeting the attached DNA to HepG2 cells. These naturally occurring proteins may be better for DNA-binding, due to the natural ability to compact DNA as well as promoting the function of nuclear targeting.

The most important step in the manipulation of the proteins or peptides for attachment to the DNA is the correct coupling of the DNA-binding agent to these components, ensuring that their function (i.e. receptor binding, endosomal lysis, etc.) is maintained. There are several different linkages that can be generated by different chemicals. The water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), has been used to link ASOR to PLL (3,20). This carbodiimide results in the formation of a covalent bond between the carboxyl groups of ASOR and the amino groups of PLL. The chemical 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester (SPDP), converts terminal amino groups into sulfhydryl groups and has been used to link Transferrin to PLL by the formation of a disulfide bond (5). A third mechanism of attachment that has been used is the interaction between biotin and streptavidin. The binding of these molecules results in one of the highest dissociation constants in nature and has been used for coupling epidermal growth factor to PLL and adenovirus to PLL (8,25). Other linkages, such as an ethidium homodimer or the Gal4 protein, have been used to form a link directly to DNA (34,38). In the instance where Gal4 was used, Uherek *et al.* showed that a ligand or component could be added on to the polyplex by incorporating the Gal4 protein into the vector. The presence of this protein allows binding to occur to the Gal4 nucleotide sequence that can be cloned into the plasmid DNA containing the therapeutic gene. Unfortunately in these last two examples, polycations are still required to mediate DNA compaction. However, these examples indicate that the method of linking the components of the polyplex can also evolve into simpler, synthetic constituents. Overall, there is

no limit to the modification of the components involved, as long as function is maintained. Accordingly, there is no limit to the type of DNA-binding agent that can be used, as long as the integrity of the nucleic acid is maintained.

6. NUCLEIC ACIDS AND *IN VITRO*/IN VIVO APPLICATIONS

The majority of research that has been done with nucleic acids, has involved the use of plasmid DNA, as it is easily manipulated and produced in large quantities. Since most of the molecular conjugates have been coupled to DNA by ionic interactions, the initial work with plasmids has focused on developing conditions that allow for efficient formation of the Protein/DNA polyplex. Original work by Wu *et al.* showed that Protein/DNA polyplexes could be made under concentrated conditions ([DNA] = 87 nM), using a step dialysis protocol that allows a change in salt concentration from 2 M to 0.15 M (3,4). Although polyplexes can be formed by this procedure, a certain amount of the Protein/DNA polyplex precipitates because of the high DNA and PLL concentration. However, Wagner *et al.* have shown that polyplexes can be formed efficiently in dilute DNA concentrations ([DNA] = 3 nM) in 0.15 M salt without precipitation of the DNA (5). Unfortunately, this low DNA concentration does not allow for use in many *in vivo* applications and has shown greater utility with *in vitro* and ex vivo approaches. As a result, the search still continues to identify conditions that result in efficient polyplex formation, such as sufficient DNA concentrations for *in vivo* administration while maintaining the size, charge and integrity of the polyplex.

Although this has required polyplexes should be formulated as "pharmaceutical compounds", the simple method by which Protein/DNA polyplexes can be generated, results in essentially no limit on the type and size of the nucleic acid that can be delivered. As mentioned, the primary type of nucleic acid that has been delivered thus far is circular DNA plasmids that have ranged in size from several kilobases (kb) to 48 kb in length (25). This large plasmid DNA was delivered by a Transferrin/DNA polyplex without a loss of delivery efficiency, when compared with polyplexes made with a smaller plasmid carrying the same reporter gene. More recent work has shown that much larger pieces of DNA know as Bacterial Artificial Chromosomes or Yeast Artificial Chromosomes, can be delivered by this vector as well (39). This suggests that this type of delivery vector has essentially no size limitation. Other types of nucleic acids such as antisense oligonucleotides have also been used in combination with a liver-specific delivery system to allow for better targeting of the oligonucleotide (40).

Since this delivery vector is easily manipulated to use any size plasmid, any therapeutic gene can be tested for expression in a particular cell type. Depending upon the ligand used, the molecular conjugate can deliver plasmid DNA to a variety of cells *in vitro* and with variable levels of transduction and expression. This is probably dependant on receptor expression, which varies between cell types. However, transduction efficiencies as high as 100% can be achieved for cells such as primary hepatocytes (20). This efficient delivery has been shown *in vitro* with an ASOR/DNA polyplex, in which the phenylalanine hydroxylase (PAH) gene, which encodes the PAH enzyme deficient in phenylketonuria, was completely replaced in primary mouse hepatocytes lacking PAH activity (20). Other experiments have shown that a plasmid expressing the gene for factor IX was expressed at high levels after delivery to primary hepatocytes, suggesting the potential correction of the disease phenotype associated with hemophilia (21). The Transferrin/DNA polyplexes have now been incorporated into a clinical protocol for the *ex vivo* transduction of melanoma cells with cytokine genes for the immunological rejection of melanoma cells (41).

The *in vivo* applications of Protein/DNA polyplexes have been limited thus far, with delivery occurring to the lung and liver. A Transferrin/DNA polyplex coupled to adenovirus and delivered to the lung epithelium by intratracheal administration resulted in less than 1% of the cells transduced (24). As mentioned, an ASOR/DNA polyplex has been used to achieve efficient gene delivery to the liver, when accompanied by a partial hepatectomy (42). As an example, when the delivery of an ASOR/DNA polyplex was accompanied by partial hepatectomy, the levels of albumin after introduction of an albumin-expressing plasmid reached 34μg/ml in the blood of analbuminemic rats (42). More recently, Perales *et. al.* have shown that DNA delivery by a galactose conjugate to the liver, can occur without partial hepatectomy by generating Protein/DNA polyplexes that are very small (10-12 nm in size) (43). Other targets for Protein/DNA polyplexes have been lung cancer cells that over-express the EGF receptor, using an EGF/DNA polyplex (8). Cook *et al.* have also shown that a DNA polyplex attached to adenovirus can deliver a toxin gene to tumor cells *in vivo* while Nguyen *et. al.* have shown that an adenovirus/PLL conjugate can mediate efficient delivery and tumor suppressor p53 expression in solid tumors (12,13,44).

However, a recurring problem with Protein/DNA polyplexes has been the transient levels of expression. This deals specifically with the genetic structure of the plasmids that have been delivered. Several groups have identified that the delivered plasmid DNA remains episomal and does not integrate (17). Most plasmids used thus far have no sequences to promote episomal maintenance or replication and thus the cells have no need to maintain the plasmids. As

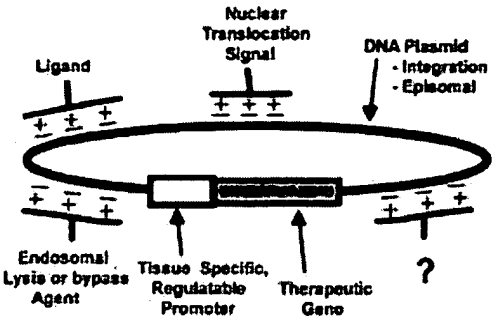

Figure 3. The general structure of a future non-viral targeted DNA delivery vector based on the use of molecular conjugates.

a result, new plasmids must be developed to address this problem, but since Protein/DNA polyplexes can be generated very easily, the testing of these plasmids will be greatly simplified. As these questions as well as others are addressed, improved Protein/DNA polyplexes will be created allowing for more efficient *in vivo* administration and utilization.

7. MOLECULAR CONJUGATES VS. OTHER DELIVERY SYSTEMS

In general, molecular conjugates and Protein/DNA polyplexes could potentially be a much more versatile gene delivery vector than vector systems that are currently available. This vector has the following properties that have been reviewed: 1) no need for packaging cell lines and as a result the vector can be used for the quick analysis of many plasmids or different types of nucleic acids; 2) the ability to target nucleic acids to specific types of cells, resulting in high level transduction of either dividing or non-dividing cells; 3) the potential lack of a viral component and therefore safe for the recipient; 4) no limitation on the size or type of the nucleic acid that can be delivered; and 5) the potential to become completely synthetic, allowing for simple and cost effective development of the delivery vector.

A true comparison between Protein/DNA polyplexes and other vectors that are capable of gene delivery for gene therapy is clearly beyond the scope of this review. However, it is clearly understood that viruses have developed the most efficient methods of gaining entry into cells as well as enhancing expression of the delivered nucleic acid. The most utilized viral vectors thus far have been: 1) recombinant retroviral vectors based on the moloney murine leukemia virus; 2) recombinant adenoviral vectors based on adenovirus serotype 5; 3) adeno-associated virus; and 4) herpes simplex virus. Overall, these viral vectors suffer from several major limiting factors: 1) all require packaging cell lines to allow for production of replication defective virus, which for retrovirus and adeno-associated virus can lead to low viral titers; 2) all have limits that affect the total amount and type of nucleic acid that can be packaged; 3) all still require many tests to ensure safety and lack of toxicity (both cellular and genotoxicity); 4) all lack the ability to target the nucleic acid to a specific cell type; and 5) all lack the ability to become completely synthetic. However, these viral vectors have unique characteristics for use in gene therapy. Retroviral vectors have the ability to integrate into the host cell genome, resulting in long term gene expression (45,46). However transduction of cells occurs predominantly in actively dividing cells (47). Recombinant adenoviral vectors have the ability to achieve much higher levels of transduction than retrovirus, especially in non-dividing cells (48,49). However, the gene expression is transient and may be due to an immune response generated against infected cells because of low level expression of adenoviral proteins (50). Adeno-associated virus has been engineered so that the complete viral genome has been removed, except for the terminal ITR that are involved in integration. The wild-type form of the virus specifically integrates at chromosome 19 at the same location, but it is unclear as to whether the recombinant form is capable of the same process (51). Herpes simplex viruses have shown the best ability to infect brain cells as the natural tropism for this virus is the brain (52). While cell specific targeting is not present in the natural form of these vectors, recent work has identified that targeting can be accomplished. Retroviral vectors have recently been modified to contain a chimeric envelope and thus targeting to breast cancer cells has been achieved (53). Adenoviral vector targeting has been accomplished through the use of chimeric antibodies that recognize both the fiber protein of the virus and the receptor that is being targeted (54). More recent work has used viruses containing modified fiber, which functions to redirect the virus to other membrane proteins (55). In the instances where genetic manipulations have been performed on the viruses, a loss of viral titer has been seen to occur.

Non-viral vectors such as Protein/DNA polyplexes, liposomes, and the mechanical delivery of naked DNA have been created to deliver nucleic acids without the aide of viruses and the their potential limitations (56-58). The latter two methods have many of the same characteristics as Protein/DNA polyplexes, but still suffer from the lack of tissue and cell specific targeting. Liposomes, which utilize lipid/nucleic acid complexes or "lipoplexes" to deliver the nucleic acid into the cytoplasm of the recipient cell, normally lack the ability to target specific cells. However, different forms of lipids, such as glycolipids, can be used to target specific organs such as the liver and more recent work has shown that the ligand can be attached to the lipid for targeting (59,60). However, the presence of the lipid component in the lipoplex can result in non-specific uptake by the reticuloendothelial system, causing a loss of targeting specificity. The liposomes also suffer from variable levels of transduction, but have been used for limited gene delivery both *in vitro* and *in vivo* (61,62). The mechanical delivery of naked DNA can be done by either direct injection into the target organ or attachment of the DNA to gold particles, which are then delivered to tissues by high-velocity bombardment (57,58). The injection of naked DNA into muscle has led to efficient DNA delivery and expression *in vivo* (57,63). However, this method of delivery seems to work primarily with the muscle and results in only cells near the injection site acquiring the DNA. The delivery of DNA by particle bombardment has also shown expression in organs such as the liver, but suffers from the lack of targeting, the inability to transduce a large number of cells, as well as the need for a surgical procedure to allow access to the tissue (58).

This comparison is not inclusive and is not meant to state that Protein/DNA polyplexes are not without limits as this vector does suffer from transient levels of expression, variability in transduction potential immunogenicity, limited

safety testing *in vivo*, and thus far, limited use *in vivo*. However, it is clear that the simplicity of the Protein/DNA polyplex and its easy manipulation should allow for these factors to be addressed much more easily than the problems associated with other vectors.

8. FUTURE DIRECTIONS

The development of molecular conjugates as a delivery vector has resulted in the creation of a simple, non-viral vector for the targeted delivery of nucleic acids into specific cell types. This system allows for quick analysis of nucleic acids, expression vectors, and therapeutic genes *in vitro* and potentially *in vivo*, since the time that would be involved in the generation of recombinant retroviral and adenoviral vectors is not present. Essentially, the development of this delivery vector has resulted in the creation of a "synthetic virus", that has the capability of targeted delivery. As a result, this vector can easily incorporate components that are important for delivery and are related to viral functions that aide in this process (figure 3). Future work will utilize this aspect; addressing problems of transient expression by developing integration and episomal maintenance plasmids based on viral systems, utilizing viral nuclear translocation signals for enhanced nuclear delivery and gene expression, as well as utilizing other properties of viruses. Crucial to the further development and use of this delivery vector will be the identification of a universal endosomal lysis or bypass agent based on either viral, bacterial, or synthetic components. As this vector matures it may also be possible to combine many of these components into one chimeric protein or peptide having multiple functions. The further manipulation of this system should also result in tissue specific and regulatable expression systems resulting in the addition of another level of specificity. As a result, there may be no limit as to the type of therapeutic gene that can be used, as well as the ligand that can be used for targeting. At this point in time, it is clear that this delivery vector can incorporate any protein or peptide that can add to the utility of this vector, resulting in a much greater use of molecular conjugates and Protein/DNA polyplexes not only *in vitro* but more importantly for the *in vivo* applications of gene therapy.

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Abbreviations: ASOR, asialoorosomucoid; CAT, chloramphenicol acetyl-transferase; PLL, poly-L-lysine; PAH, phenylalanine hydroxylase; CMV, cytomegalovirus; enhancer/promoter, DNA, deoxyribonucleic acid.

Send correspondence to: Richard J. Cristiano, Ph.D., Section of Thoracic Molecular Oncology, Department of Thoracic and Cardiovascular Surgery, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Box 109, Houston, Texas 77030. Tel:(713)-794-4036 Fax: (713)-794-4669, E-mail address: rcristia@notes.mdacc.tmc.edu